THESIS

THE EFFECTS OF CORN ON MICRORNA EXPRESSION WITHIN HORSES

Submitted by

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In partial fulfillment of the requirements

For the degree of Master of Science

Colorado State University

Fort Collins, Colorado

Spring 2021

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ABSTRACT

EFFECTS OF CORN ON MICRORNA EXPRESSION WITHIN HORSES

Nutrition has been shown to play a major role in the health of horses in all life stages and levels of work. In recent years the prevalence of equine obesity has increased as more horses are kept in stalls with lower workloads, while receiving high energy and calorically dense feeds like grain, many of which contain corn, in addition to forage. The increase in equine obesity has been accompanied by more cases of metabolic diseases developing, often linked to poor nutrition and diets high in non-structural carbohydrates (NSC). Although more cases of metabolic disorders are emerging there currently are no good biomarkers to diagnose these diseases or identify horses on diets providing them with poor nutrition. Diets high in NSCs have been linked to insulin resistance and laminitis within horses, two of the main components of Equine Metabolic Syndrome (EMS), however nutrigenomic studies looking at the interaction of diets high in NSCs on gene expression, specifically through the regulation of endogenous microRNAs (miRNA) are rare. Recent research on mice and human models has demonstrated the large impact diet has on levels of miRNAs within the body and mRNA targets for these miRNAs resulting in the regulation of gene expression, in addition to identifying miRNAs in circulation that can be used as biomarkers for obesity, type 2 diabetes, and metabolic syndrome. Research has also demonstrated the ability of diet-derived exogenous miRNAs to be absorbed from the digestive tract, appear within circulation, and be taken up by various tissues throughout the body. Diet-derived miRNAs specifically from plants have been detected in tissue and circulating within the blood suggesting the possibility of cross-species gene regulation, but the exact role these

miRNAs play physiologically is still unknown. miRNAs are small non-coding molecules that affect post-transcriptional gene regulation and RNA silencing by translational repression or degradation. Previous research revealed that some plant miRNAs could be identified in equine serum exosomes and tissues but was not able to identify a corn specific miRNA within any equine samples.

We first hypothesized that diet-derived corn miRNAs can be detected in equine serum and muscle after corn supplementation. For this study twelve mares were blocked by weight and BCS and assigned to one of two treatments (n=6/group): 1) control, (basal diet: 20 lbs./head/d of chopped mixed alfalfa-grass hay and ad libitum mixed grass hay), 2) basal diet supplemented with 1 lb./d steam flaked corn. Muscle biopsies of the *Gluteus medius* and serum samples were collected from all horses on d0 and d28. Samples were analyzed using real-time RT-qPCR for 3 plant miRNAs. Our results revealed the presence of plant miRNAs in equine total serum and skeletal muscle. Our results also revealed the level of plant miRNAs, including the corn specific miRNA, within circulation vary after ingestion, suggesting plant miRNAs are capable of being taken up by equine tissues. These results are important for understanding how physiological processes may be impacted by diet-derived plant miRNAs. Moreover, these results suggest plant miRNAs could potentially serve a therapeutic role in helping to regulate endogenous gene expression in addition to the nutrients being provided by ingestion. The large impact diet can have on equine health and the association between diets high in NSC and insulin resistance, caused us to be interested in the effects a diet supplemented with corn would have on endogenous miRNAs within the horse. We hypothesized that supplementing horses with corn would alter the endogenous miRNA profiles within both serum and skeletal muscle. For this objective, we utilized the same serum and muscle samples collected for the feed trial horses as

the plant miRNAs. Samples were analyzed using real-time RT-qPCR for 277 endogenous equine miRNAs. Our results showed 13 differentially expressed (P<.05) miRNAs in equine serum after 28 days of corn supplementation. Six of these miRNAs (eca-mir16, -4863p, -4865p, -126-3p, -296, and -192), were linked to obesity and/or metabolic disease. Within skeletal muscle, our results showed three miRNAs differentially expressed (P<.05) and three miRNAs with a trend toward differential expression (.05<P<.1) after 28 days of corn supplementation. The differently expressed (P<.05) miRNAs in muscle (eca-mir1515p, eca-mir106b, and eca-mir133a) were all associated with obesity or muscle insulin response, while two of the miRNAs with a trend towards differential expression (.05<P<.1), ecamir-10b and eca-mir129a5p, were associated with diabetes/hyperglycemia and glucose metabolism respectively. This data indicates that diet does influence levels of endogenous miRNAs.

The results of our study indicate that diet-derived plant miRNAs can appear in circulation, be taken up into tissues, and hold the potential to regulate endogenous genes. This study also provides more information on how a diet high in NSCs, like diets containing corn, alters levels of endogenous miRNAs within serum and tissue and helps to better understand the relationship between diet, health, and disease within horses.

ACKNOWLEDGMENTS

I have received constant support from many people throughout my Master's Degree. I would first like to thank my advisor, Dr. Tanja Hess, for her continuous support throughout my entire academic career at Colorado State University. Thank you to my committee members Drs. Jason Brummer, Stephen Coleman, and Gabriele Landolt, who dedicated a portion of their valuable time to help me complete this degree. I would also like to thank Terry Engle for finding the time in his very busy schedule to help me throughout my master's program and entertaining my anxious ramblings at times.

Thank you to my family for continuously supporting me throughout this project and my life. I would not be where I am today without all the love and support that has been in my life.

Thank you to my dear friends Rowan Seabolt, Nicole Tillquist, Meghan Thorndyke, and the MacNeil twins for all of your support and help throughout this project.

I would like to especially thank all the graduate, undergraduate, and veterinary students that assisted me in feeding horses and collecting samples. This amazing group of volunteers willingly gave up the opportunity to sleep in during their summer and showed up every morning with a smile and often a corny joke or two.

My combined program was sponsored by a donation from the Y-Cross Ranch, whom I would like to say a special thank you to as I have received scholarships from them as an undergraduate, graduate, and veterinary student allowing me to continue my pursuit of knowledge.

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INTRODUCTION

The diet and feeding behaviors of horses today are dramatically different from those of their ancestors and tailored to human convenience, not physiologic make-up. Research has shown that equine performance is heavily influenced by many factors including genetics, nutrition, metabolism, exercise, and stress. The smaller two region stomach and lack of gall bladder in the equine make them more suited physiologically to periodic grazing throughout the day than the typical two concentrated meals per day that are implemented at most equine facilities. Many equine rations contain energy supplements like corn, which is a high energy feed and low cost to provide horses with the energy necessary to meet performance expectations. Corn contains starch, a highly fermentable carbohydrate, which is known to contribute to laminitis. Corn and other non-structural carbohydrates have been linked to a variety of metabolic issues including laminitis, insulin resistance, and obesity (Pollitt and Visser, 2010). These metabolic issues when displayed together may indicate the horse has Equine Metabolic Syndrome (EMS), a similar condition to diabetes mellitus type 2 in humans. These issues and conditions are not yet fully understood and investigations into the dietary role in the regulation and expression of genes are being conducted, as well as attempting to identify reliable markers to use in the diagnosis of these conditions. The role of small RNA molecules called microRNAs (miRNAs) in gene regulation and as disease markers are subject to recent and current research (Nulton, 2014; Santos et al., 2018), as well as the presence of plant-derived miRNAs in mammalian serum (Zhang et al., 2012).

MicroRNAs are small non-coding molecules about 22 nucleotides in length that affect post-transcriptional gene regulation and RNA silencing found circulating freely in blood or

within micro-vesicles (Zhang et al., 2012). The miRNAs are partially complementary to a few target mRNAs which they inactivate (Zaiou et al.,2018). Nearly all cell functions are thought to have miRNAs involved in their regulation, with miRNAs circulating in the blood thought to have a large role in the regulation of cells from a distance and cell communication. Interest in miRNAs and their roles within diseases and cellular processes has led to increased research in both healthy and diseased individuals in many species (Garcia-Elias et al., 2017). Recent studies have found a connection between specific miRNAs in humans and higher rates of obesity and metabolic diseases like type 2 diabetes. Some miRNAs in horses have also been linked to metabolic disease, obesity, and insulin resistance (Santos et al., 2018). The trend of increasing metabolic issues within horses can be linked to the shift in their diets from the natural 16 hours a day of grazing to two meals a day with increased non-structural carbohydrate (NSC) content.

This study is one of the first to investigate the impact of diet on the profiles of endogenous miRNA levels and the presence of diet-derived exogenous plant miRNAs in horses. This study will add to the body of work within other mammals investigating the idea that miRNAs can be taken up from the diet and how diet impacts miRNAs in different species. Specifically, the effects of a corn supplemented diet on endogenous miRNA levels compared to a control diet (grass and alfalfa only) will be examined.

CHAPTER I: LITERATURE REVIEW

MICRORNAS

MiRNAs are short non-coding RNAs ranging from 18-25 nucleotides in length responsible for inhibiting translation and degrading mRNAs by binding to the 3' untranslated regions, coding sequences, or 5' untranslated regions (Alemida et al.,2011). A large portion of the human genome is affected by miRNAs illustrating their large impact upon gene regulation within the body (Garcia et al, 2017). Many biological functions are influenced by miRNA regulated genes. The first miRNA identified was *lin-4* located within the genes controlling larval stages in the nematode *Caenorhabditis elegans* in 1993. The second miRNA to be discovered, let-7, was found in 2000 and contributes to larval stage regulation in nematodes as well (Alemida et al., 2011). The discovery of let-7 and its conservation across species led to thousands more miRNAs being discovered in a variety of different species and the identification of more universally conserved miRNAs. The miRNA functions within plants and animals have been found to slightly differ. Plant miRNAs affect RNA cleavage and degradation while animal miRNAs inhibit translation and alter transcription (Taylor et al., 2014). In addition to their gene silencing activity, miRNAs have also been found to interfere with regulatory proteins through decoy activity. Decoy activity is when a miRNA binds to a region, instead of the normal gene, an example being when miR-328 was found to independently bind to the non-seed region of hnRNP E2 and prevented the binding of *CEBPA* with the protein (Alemida et al., 2011). The specific function of many miRNAs has yet to be discovered and new research continues to identify new functions.

Biogenesis

MiRNA coding genes most frequently occur within the introns or intergenic regions of protein-coding genes. Intergenic regions are located in between genes, while introns are located within a gene and removed before protein assembly. For mammalian miRNAs, the miRNA coding gene is transcribed by RNA polymerase II (Lee et al, 2004) or in some cases RNA polymerase III (Gu et al, 2010). RNA polymerase II is the catalytic portion of the enzyme complex responsible for transcribing DNA in mRNA (Cramer, 2004), suggesting miRNA is an essential regulatory component for normal cellular function and can be derived from loci throughout the genome. Within the nucleus creating primary miRNAs (pri-miRNAs). Pri-miRNAs have a 5' cap and poly-adenylated 3' and are converted by RNAse III Drosha and Pasha proteins within the nucleus to pre-miRNA (pre-miRNA) which are 60-100 nucleotides in length. The pre-miRNA is then transported into the cytoplasm by an exportin-5 dependent Ran protein and converted by the Dicer-1 enzyme and PACT proteins into a double-stranded miRNA duplex that is shorter in length than the pre-miRNA. The duplex is then unwound by helicase into a single-stranded mature miRNA ranging from 15-22 nucleotides in length. One strand of miRNA is degraded, while the other strand is incorporated into an Argonaute (AGO) protein complex to create the miRNA-induced silencing complex (miRISC), which acts as a mediator for gene silencing (Alemida et al., 2011). The biogenesis of mammalian miRNAs is illustrated in Figure 1. Some miRNAs contain additional sequences that control their location within the cell of the animal, for example, miR-29b contains an extra six nucleotide terminal motif that directs it back into the nucleus after formation and maturation (Hwang et al., 2007).

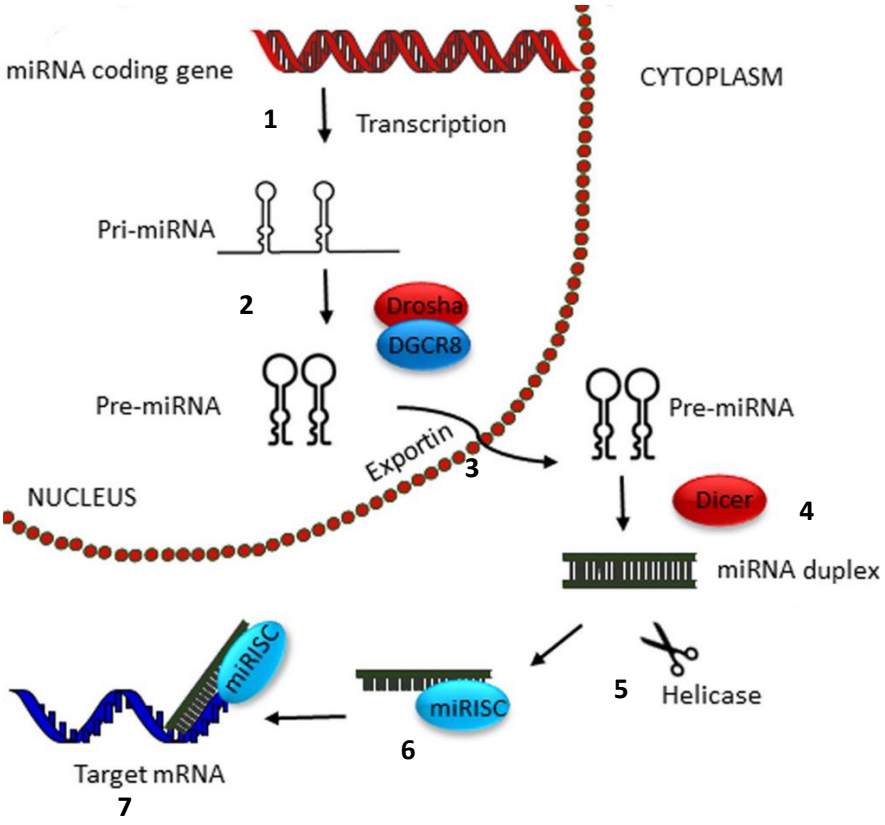

Figure 1: mammalian microRNA synthesis and mechanism of gene regulation.1. MicroRNAs are transcribed by RNA polymerase III (not shown in the figure) and form pri-miRNAs, one form of RNA precursors. 2.Pri-miRNAs are processed in the nucleus by Drosha and DGCR8, resulting in pre-miRNAs. 3. Pre-miRNAs are transported into the cytoplasm via exportin-5. 4. Once in the cytoplasm, Dicer continues to process the pre-miRNA forming a miRNA duplex with two complementary short miRNA sequences. 5. Helicase breaks apart the miRNA duplex into individual molecules. 6. One miRNA sequence is integrated into the RISC complex via an AGO protein (not pictured), while the other strand is degraded. 7. At this point, miRNAs can exert their regulatory effects on messenger RNAs (mRNAs) via mRNA cleavage or translational repression. Adapted from: Saif and Emanueli, 2014.

The biogenesis of plant miRNAs is very similar to that of mammalian miRNAs, but a few different plant enzymes are utilized within the process in place of their mammalian counterparts (Figure 2). miRNA coding genes are translated to pri-miRNAs within the nucleus by RNA polymerase II. The pri-miRNAs are then converted to pre-miRNAs through interactions with DCL1 (dicer-like 1), dsRNA-binding protein 1 (dRB1), and Hyponastic leaves1 (HYL1) which cleaves the RNA. The pre-miRNA is acted on a second time by DCL1 and HYL1 to form the mature miRNA duplex within the nucleus still. Before exiting the nucleus, the mature plant miRNA duplex is methylated by HUA Enhancer 1 (HEN1), an sRNA-specific methyltransferase,

giving the mature plant miRNA the defining characteristic of a 2'-O-Me modification on 3' end. The mature methylated miRNA is then transported into the cytoplasm by HASTY, the plant ortholog to Exportin-5. Within the cytoplasm plant miRNAs, like mammalian miRNAs, combines with an AGO protein (AGO1) to form the miRISC, however unlike mammalian miRNAs plant miRNAs need ATP and Hsc70/Hsp90 to help with the formation of the miRISC (Iwasaki et al., 2010; Nakanishi, 2016).

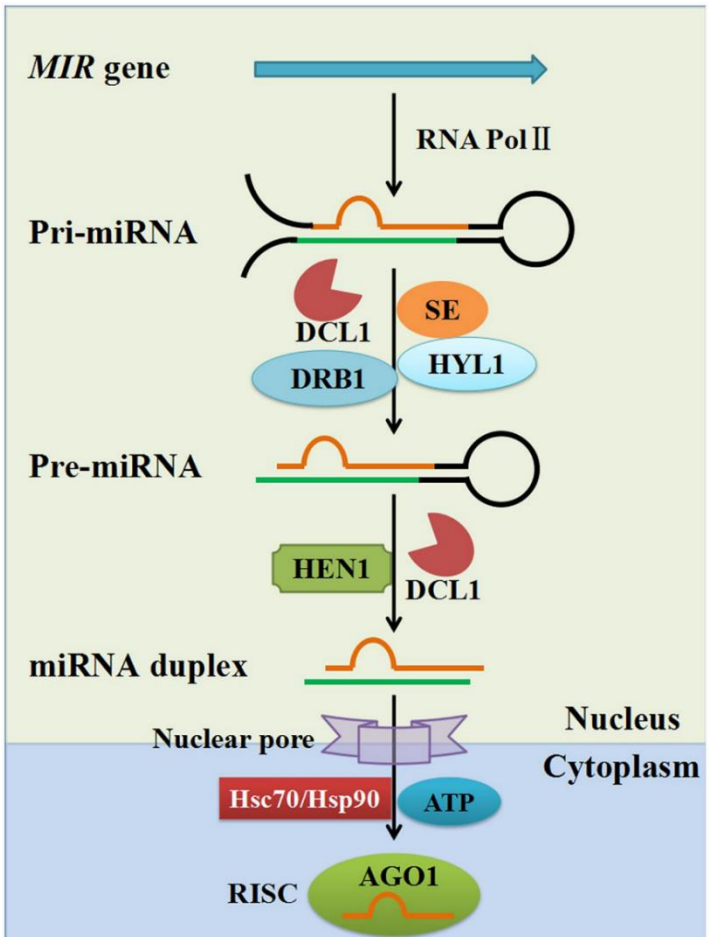

Figure 2: The biogenesis of Plant miRNAs. Plant pri-miRNAs are produced from MIR genes by RNA polymerase II (Pol II). Pri-miRNAs are cleaved into pre-miRNAs by DCL1, DRB1, and HYL1. Pre-miRNAs are cleaved into miRNA duplexes by DCL1 and HEN1. MiRNA duplexes are methylated by HEN1 into mature miRNA duplexes and are exported to the cytoplasm through the action of HASTY. One strand is then degraded and the other is then loaded onto an AGO protein with the help of Hsc70/Hsp90 and ATP, to form a miRISC which influences gene silencing. Original Source: Liu, S. et al., 2017

MiRNA modes of action

MiRNAs are a central component of RNA interference, a biologic process within which a targeted mRNA is silenced by an RNA molecule resulting in the inhibition of translation or gene expression. RNA interference begins when the Dicer enzyme cleaves the pre-miRNAs into miRNA duplexes before helicase breaks the duplex into 2 separate RNA sequences. One of the sequences degrades while the other miRNA sequences bind to an RNA-induced silencing complex (RISC), to form the miRISC complex that is ultimately responsible for the destruction of the target mRNA resulting in gene silencing (Sontheimer, 2005). The exact mechanism to achieve gene silencing differs between plant and mammalian miRNAs. Mammalian miRNAs target the untranslated regions of mRNAs and bind to the target region with the standard base pair matching conventions. The mechanism of gene silencing is determined by the degree and nature of complementary nucleotides within the target region compared to the miRNA. Cleavage of the target region by Ago2 endonucleases requires extensive near-perfect matches of base pairs, in contrast, translational inhibition requires multiple sites of complementary sequences with minimal base-pairing in each site; Hence, why translational inhibition is the more common mode of gene silencing for mammalian miRNAs (Bartel 2004, Yekta et al., 2004). Within mammals, the entire miRNA may not match the target mRNA completely resulting in RNA hybrids that contain bulges that alter the specificity and could impact the gene silencing ability of the miRNA (Brennecke et al., 2005). Plant miRNAs achieve gene silencing mainly through the process of mRNA cleavage, however, they can also do translation inhibition (Broderson et. al, 2008). Plant miRNAs unlike mammalian miRNAs target the coding regions of mRNAs and cleave them apart. As a result of cleaving the mRNA apart, it decays, an irreversible process,

while translational inhibition can be reversed. It is generally accepted that multiple miRNAs can bind to the same targets on genes and miRNAs can have multiple targets they can influence. This means that plant and mammalian miRNAs could potentially target some of the same genes resulting in silencing of the same gene, highlighting the importance of understanding where miRNAs are located within the body and how they can move throughout the body via circulation.

Extracellular miRNAs

The majority of identified miRNAs have been found within cells, but the presence of miRNAs outside of the cellular environment has also been well identified (Zhu et al, 2011, Lechhi et al, 2017). The ability of miRNAs to enter the extracellular environment and contribute to cell-to-cell communication occurs through secretion through five different mechanisms: microvesicles, exosomes, apoptotic bodies, high-density lipoproteins (HDL), or Ago protein complexes (Makarova et al., 2016, Sohel, 2016). Microvesicles are formed through the direct process of budding off the plasma membrane and fission of the two sides to encapsulate the contents now in the extracellular environment, they can be differentiated from exosome by surface markers and size (Lee et. al, 2012). Exosome also involves budding to transport their contents from the inside of the cell to the outside, however, they are completely formed within the cell by invagination of the cell wall and must receive a signal to bud off into the extracellular environment (Johnstone, 2006). Another potential method of transporting miRNA in the extracellular environment are apoptotic bodies, which are the largest of the possible methods and are a result of cell death via apoptosis. Apoptotic bodies are formed towards the end of the process of apoptosis and can contain a variety of nucleic acids in addition to miRNAs, the exact

impact and mechanism of miRNAs carried by apoptotic bodies are not currently known and future research is needed to better determine how much of a role this particular method of transportation impact cell to cell communication (Soehl, 2016). HDL is usually associated with the digestive and metabolic process, but its high affinity for water-insoluble compounds makes it capable of attracting and carrying miRNAs throughout the body and facilitate communication between cells all over the body. Unlike microvesicles, exosomes, and apoptotic bodies purified HDLs only contain small non-coding RNAs (Vickers et al., 2011). The fifth method potentially responsible for extracellular miRNAs has already been mentioned as AGO protein complexes form RISC complexes with miRNA, AGO2 is part of the RISC and has been found with miRNA in cell culture media (Turchinovich et al., 2011). A study by Arroyo et al. demonstrates that only about 10% of cell-free miRNA within human plasma was released through microvesicles, with potentially up to 90% of the cell-free miRNA found within circulation we found to co-fraction, using size-specific chromatography, with ribonucleo-protein complexes like the AGO family (Arroyo et al., 2011). Looking at studies identifying the presence of extracellular miRNAs the sample type, method of extraction, quantification tests, and normalization procedures all seem to impact the ability to identify miRNAs within the extracellular environment, making it currently challenging to definitively identify which of the five potential methods has the most impact upon cell-to-cell communication and gene regulation.

UPTAKE OF DIETARY MIRNA

The uptake of miRNAs from the diet is not currently fully understood and is highly debated. The first studies to show the possibility of diet-derived miRNAs were conducted with rice, a common and staple food throughout the world. Many studies have found evidence of diet-

derived plant miRNAs within mammals, however, many contradicting studies were not able to find plant miRNAs within mammals, with the reliability of detection and biologically significant levels being the largest issues. The low repeatability of identifying dietary miRNAs in circulation may be due to the short window of detectability, because of their rapid clearance and degradation by nucleases. When miRNAs were directly injected into the vein, they were completely cleared from circulation within three hours (Yang et al., 2015). The detection of miRNAs within circulation suggests nuclease resistant forms of miRNA exist and remain in circulation for longer periods than those that are not resistant (Yang et al. 2016).

MiR2911 is found within plants and was found in increased levels in the circulation of mice fed diets containing various plants (Yang et al., 2015; Yang et al., 2016). Mice receiving feed with ground honeysuckle had miR2911 expression 39-fold higher at the end of a seven-day feeding trial compared with before the trial (Yang et al., 2015). This miRNA was also found to be the most stable during digestion when compared to miR168, a plant miRNA that has also been found in circulation (Zhang et al., 2012), and an artificial mRNA (Yang et al.,2016). In contrast, corn specific miRNA was not found in circulation when fed to mice with a water gavage, likely uptake into circulation may be inhibited by excessive and early degradation during the process of digestion (Huang et al., 2018). Corn specific miRNA was also not found in equine circulation six hours after consuming 1 pound of corn, however, a common plant miRNA, miR 156a, was identified within serum exosomes at this time point as well as within the tissues (Nulton, 2014). Exosomes are endosome derived extracellular lipid-membrane bound vesicles that transport proteins and RNAs between cells. One theory of how miRNAs from food exit the lumen of the intestines and into other systems within the body involves the miRNA being encapsulated by nutrient particles from the food which are then absorbed into the lymphatic and

circulatory systems through the digestion process (Chan and Snow, 2017). This theory also states that potentially miRNA from the diet gets from the lumen of the gastrointestinal tract into circulation and subsequently various tissues by being packaged within exosomes or ribonucleoprotein (RNP) complexes, like HDL. Once packaged the RNP complexes or exosomes cross the epithelium by transcellular transportation, paracellular transportation, or immune conveyance mechanisms. Once through the barrier, they can go into the circulatory system, lymphatics system, or nearby connective tissue cells. Within the circulatory system, the exosomes and/or the RNP complexes are carried throughout the body for possible uptake by a wide variety of tissues (Chan and Snow, 2017). This process is illustrated in Figure 3.

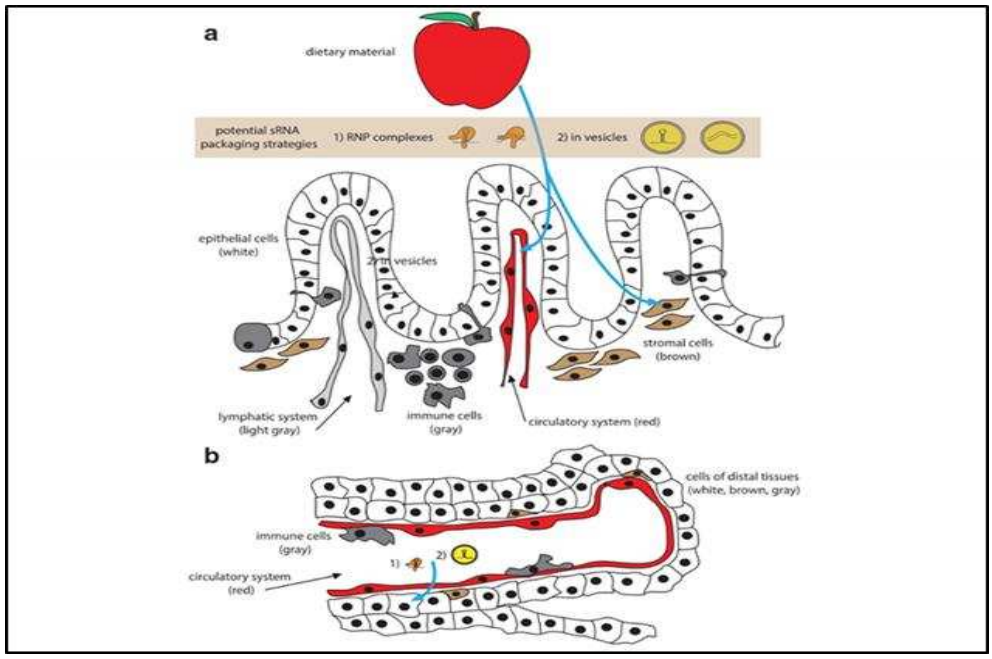

Figure 3: Diagram of a possible method of Dietary miRNA uptake from the digestive tract. To potentially impact gene expression within a mammal the miRNAs from the diet must be absorbed from the digestive tract into circulation or intestinal tissues. A) miRNAs from the diet (potentially packed in 1) RNP complexes or 2) within a vesicle) cross the epithelial barrier (white cells) via transcellular, paracellular, or immune cell conveyance (gray). Once through the epithelial barrier, the miRNAs are likely taken up by stromal cells (brown), neighboring cells, lymph systems (light grey) or enter the circulatory system (red). B) once in the circulatory system the miRNAs could exit via many mechanisms to a wide variety of cells, tissues, and organs. The molecular level of this process warrants more studies to develop a better understanding. Original Source: Chan and Snow, 2017.

Transcellular pathways through the epithelium involve the exosome binding to the cell membrane or transported whole into the cell via protein transporters before passing through the cytoplasm and being transported to the outside of the cell on the other side. Paracellular transportation involves the exosome or RNP complex to pass between the junctions between the epithelial cells. In a normal healthy individual, there should be tight junctions between the intestinal epithelial cells. The presence of tight junctions in a healthy individual would make paracellular transport unlikely, however, in an individual that has compromised epithelium within their intestines, these tight junctions may be weakened allowing paracellular transport to occur. Within humans the epithelial barrier begins to lose its function with age and allows for some paracellular transport, this can also potentially occur with inflammation, toxins, and infection (Chan and Snow, 2017). Horses also can have a breakdown in the integrity of their intestinal epithelium allowing for paracellular transport. This equine condition has also been termed 'leaky gut' as it has been in humans. Within horses, the breakdown of this barrier can be attributed to a variety of causes including stress, ischemic reperfusion injuries (as would occur after a colon torsion), pathogens, inflammatory bowel disease, microbiota, and nutrition. Within horses, the nutritional component most often believed to cause damage to the epithelium is an overabundance of starch which can lead to acidosis which damages the intestinal lining (Stewart et al., 2017). This potential for more uptake in individuals with compromised epithelial barrier could explain why there is so much variation in the detection of miRNA uptake by individuals.

The surface of the intestines is composed of more than just epithelial cells, there are also immune cells integrated within the surface that could also play a potential role in the uptake of miRNA from within the lumen. Immune cells that populate the surface of the intestines include M cells, B cells, T cells, macrophages, and dendritic cells (Peterson and Artis, 2014). Some plant

nanoparticles have been identified that target intestinal macrophages, but they do not appear to be dependent upon miRNAs and no data shows the delivery of miRNAs to other cells via immune cell delivery(Mu et al., 2014).

Within mammals the possibility of miRNAs crossing the intestinal epithelium and becoming widely spread throughout the body is complicated and would likely require multiple rounds of ingestion, crossing the intestinal barrier, and uptake by cells to even hold the possibility of reaching distant tissues. This brings in to question the frequency and dosage of miRNAs within the food that would be needed to achieve systemic spread and uptake of these dietary miRNAs (Chan and Snow, 2017). Although there are many theories the exact mechanisms involved in the uptake of exogenous miRNA into mammalian circulation still need further exploration as current studies contradict each other and the mechanisms involved in miRNA absorption are still not fully understood.

miRNA ROLE IN DISEASE

The role of miRNA in disease has been an increasingly popular area of research since its discovery and categorization. The level of expression of certain miRNAs has been found to differ in diseased mammals versus healthy mammals. The use of miRNAs as biomarkers has largely been within patients suffering from a wide variety of cancers, viral infections, nervous system disorders, cardiovascular diseases, and metabolic diseases. Specific miRNA changes have been found for large B-cell lymphoma, lung cancer, breast cancer, liver cancer, pancreatic cancer, ovarian cancer, and prostate cancer (Wang et al., 2015). Due to the increased occurrence of human metabolic diseases like diabetes, research has been done to identify miRNAs that can

be used as biomarkers for the disease and their role as regulators of metabolic functions (Wang et al., 2015). These studies have identified many miRNAs that contribute to human metabolic diseases, with more research needed to confirm the validity of the current data.

Obesity

Obesity and being overweight occur when an individual has a high percentage of their body composed of fat. Obesity is a complex metabolic disorder characterized by an excessive accumulation of fat in various body parts (Khan et al., 2015). Within horses, fat tends to accumulate along the crest of the neck, the withers, the loin, the tailhead, behind the shoulders, and over the ribs (Figure 4), or generally distributed where abnormal amounts of fat can be found all over the body. Within horses using the Henneke body condition scoring system a score of 6-7 is considered overweight and 8-9 is considered obese, description of each score within this scoring system can be found in Appendix Table IV. Fat is made up of adipose tissue and is not just an energy-storing tissue, it also releases adipokines which help to regulate immune function, energy metabolism, reproductive status, and cardiovascular function. The most well-known and studied adipokines are leptin, adiponectin, angiotensin, and more recently resistin (Radin et al. 2009). Leptin is released from white adipose tissue and acts on the hypothalamus, which contains multiple nuclei responsible for regulating appetite and metabolism.

When an animal has an increase in fat mass, like in the case of an animal becoming obese, dysregulation in the production of adipokines occurs resulting in diseases within patients. Recent studies have begun to identify miRNAs linked to the promotion and inhibition of adipogenesis and the development of obesity, and obesity-related diseases, which could also have impacts upon adipokines (Zaiou et al., 2018). Support for miRNA's role in adipogenesis

came from studies showing that adipogenesis was altered when the DICER enzyme, which is a key factor in miRNA generation, was knocked out and decreased fatty acids were present within the body (Mudhasani, et al., 2010). Additionally, miRNAs circulating within the body have been found to change in patients with diseases like type 2 diabetes mellitus (T2DM), hypertension, hepatic injury, and atherosclerosis (Rottiers and Narr, 2012). Type 2 diabetes mellitus is a metabolic condition in humans, dogs, and cats most commonly believed to be caused by insulin resistance with the majority of individuals with this disease being classified as obese or on the top end of overweight. The *Let-7* family of miRNAs has been shown to regulate glucose metabolism in a variety of different organs, mice in which *Let-7* was knocked down showed increased glucose tolerance and a reduction of fat mass (Frost and Olson, 2011). Within horse the reports of true cases of Diabetes mellitus are rare, but Equine Metabolic Syndrome (EMS) has many similar factors involved within it. EMS is most often associated with obesity, insulin dysregulation, and laminitis signs similar to how T2DM can manifest in humans who are often obese, insulin-resistant, and can develop neuropathies in lower extremities if left unmanaged. There are currently no miRNAs within horses identified for use as biomarkers of EMS. Most Horses with EMS often are classified as obese, but the most consistent and important feature of EMS is insulin dysregulation, with laminitis being the consequence of horses with clinical EMS. The trend for horses with EMS also being obese is likely due to the decreased production of adiponectin which is associated with insulin sensitivity and a reduction in inflammation, making it inversely related to obesity and insulin resistance. (Durham et al., 2019). The reduction in adiponectin in obese individuals allows for a chronic state of low-grade inflammation to persist which also favors the development of insulin resistance.

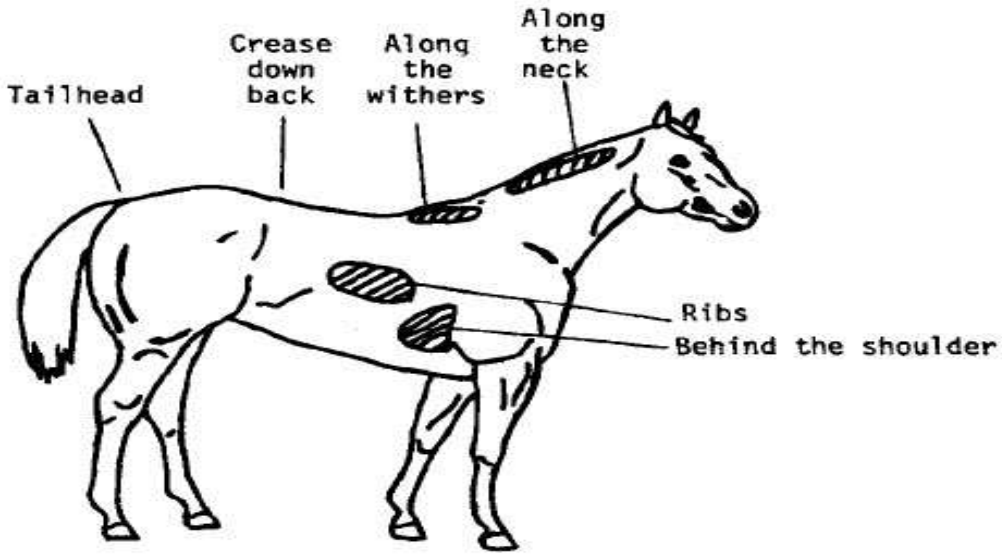

Figure 4: Areas of evaluation when body conditioning horses with the Henneke system. When body condition scoring a horse area of interest for fat deposition are along the neck, withers, ribs, tailhead, behind the shoulder, and if a crease is formed down the back. Each area should be evaluated and scored to accurately give the horse's overall score as not all horses evenly distribute fat across these areas. Source: Henneke et. al, 1983.

Insulin Resistance

Insulin resistance (IR) is a state of reduced responsiveness of tissues to insulin, regardless of if it is at normal or elevated levels within the blood; this mainly affects hepatic, skeletal muscle, and adipose cells within tissues (Bhattacharya et al. 2007, Frank and Tadros 2013). Insulin resistance is an area of special interest within horses as it not only plays an important role in EMS but also has been associated with the development of laminitis in horses (Bertin and de Laat, 2017). In the early stages of insulin resistance, the beta cells of the pancreas compensate by producing more insulin to minimize the period during which blood glucose levels are elevated. As the condition continues the insulin receptors in the tissue become more desensitized to insulin and the pancreas continues to produce waves of insulin to produce a strong enough signal to bring glucose concentration down to basal levels. There are a few mechanisms that

have been identified as contributing to the tissue insulin receptor. The first is pre-receptor abnormalities resulting in reduced insulin concentration or receptors for the tissue of interest. The second and more common one is for abnormalities in signal transduction to occur which results in IR. The abnormal signal transduction could be attributed to reduced tyrosine-kinase activity and reduced receptor phosphorylation which then causes a reduction in the phosphorylation of insulin receptor substrate-1 and decreased phosphatidylinositol 3-kinase activity. The third mechanism is abnormalities in the metabolism of glucose within cells, regulated by hexokinase and glycogen synthetase, which could be reduced by insulin-dependent uptake of glucose and its subsequent storage (Treiber et al., 2006). Hyperinsulinemia occurs when there is too much insulin within circulation for the amount of stimulus the body received and can occur intermittently or persistently (Frank and Tadros, 2013). Hyperinsulinemia doesn't only occur as a result of tissue insulin resistance it can also happen for other physiological reasons like an exaggerated response to a meal containing carbohydrates resulting in transient hyperinsulinemia with no insulin-resistant tissue present (de Laat, McGree, and Sillence, 2016). When healthy horses were infused within insulin to create a hyperinsulinemia state some individuals did develop laminitis (Asplin et al., 2007) illustrating a link between laminitis and hyperinsulinemia. This suggests that insulin resistance and hyperinsulinemia could separately contribute to the development of laminitis in horses, rather than both factors needing to be present. Laminitis is a painful condition and can have a serious impact on a horse's quality of life, there are currently no consistently effective ways to treat laminitis making prevention the best option for many horses, especially horses with EMS. Many different tests can be done to diagnose a horse with insulin resistance or hyperinsulinemia including fasting glucose tests, the euglycemic hyperinsulinemic clamp test, insulin sensitivity test, or the frequently sampled

intravenous glucose test (Bertin and de Laat, 2017), however many of these tests are time-consuming and expensive minimizing their useful impact to the industry as a whole. The development of an economical test that could identify insulin-resistant horses before they develop EMS or develop laminitis there would be a large impact on the equine industry and general equine welfare.

The profile of miRNAs endogenous to horses has recently been looked at to determine if they differ between horses that are insulin resistant and horses that are considered insulin sensitive or normal. Insulin resistant horses were found to have lower levels of five miRNAs (esa-mir-147b, esa-mir-370, esa-mir-376c, esa-mir-139- 5p, and esa-mir-1839), higher levels of three miRNAs (esa-mir-129a-5p, esa-mir-770, and esa-mir-140-3p) and lacking six miRNAs compared to the insulin-sensitive horses (Santos et al., 2018). The mir-376 family has been shown to have implications on the regulation of pancreatic beta-cells, cell growth, and could potentially serve as a predictive biomarker for obesity. Specifically, mir-376c has been found to lack expression in the pancreatic islets of rats with type 2 diabetes (Santos et al.,2018). More studies within horses should be conducted to confirm the potential biomarkers.

Laminitis

Equine laminitis can be caused by a wide range of events, most commonly the ingestion of a large amount of NSC within a short period. This large dose of NSC is usually through the ingestion of large quantities of grain or fresh grass. During the digestion of these feeds, dysbiosis occurs resulting in the release of toxins that weaken the laminae of the hoof and cause inflammation (Pollitt and Visser, 2010). In recent years the effect of long-term obesity on the incidence of laminitis has also been explored, in addition to miRNA biomarkers for this condition. Horses that are obese are in a chronic state of inflammation and have higher

mechanical loads for the laminae to support. This leads to the inflammation and weakening of the laminae over time from the strain of supporting the extra weight, in addition to hormonal changes that occur. Feeding horses NSC at low levels for a long period does not have the same detrimental effects as episodes of high NSC intake, such as grain overload, however, more research needs to be done to indicate if a diet with NSC at a low level alters the metabolism of circulating miRNAs associated with laminitis. Three miRNAs have been found to be expressed in different levels in animals displaying acute laminitis, with levels of miR-23b-3p, miR-145-5p, and miR-200b-3p being significantly higher in affected horses (Lecchi et al., 2017). The accuracy of the diagnosis of acute laminitis using the miRNA profile of horses was found to be a good indicator of acute laminitis and positively correlated with the composite pain scale and horse grimace scale that have been used to determine the severity of lameness. Although the grimace scale is a good indicator of pain, it is not completely reliable as a diagnostic tool as it becomes unreliable when looking at stills from videos or photos with no context of environmental factors that could be affecting the horse's expression (Lecchi et al., 2017). Using miRNA as biomarkers for diseases in mammals is increasing in use as more studies identify miRNA expression linked to disease.

DIETARY REGULATION OF ENDOGENOUS miRNA

Diet has a huge impact on how mammalian systems function, with many studies about the impact of different foods and quantities on health being conducted, including on how diet impacts miRNAs. Nutrients and micronutrients within diet have been linked to modifications in miRNA profiles within mammals (Garcia-Segura et al., 2013). Diets deficient in amino acids have been found to produce proteins that compete with miRNAs for binding sites, altering the

regulatory abilities of miRNAs without the levels of miRNA changing (Bhattacharyya et al., 2006). Amino acids can also cause an increase in miRNA expression, Drummond et al. found that after administering a mix of essential amino acids to human subjects there was an increase in levels of miR-1, -23a, -208b, and -499 within skeletal muscle biopsies (Drummond et al., 2009). The presence of some fatty acids within the diet has been found to alter miRNA expression. Diets supplemented with butyrate, a short-chain fatty acid produced by bacteria in mammalian colons, resulted in the upregulation of miR-10a and miR-24 which can target the HOXA1 and Notch1 genes, ultimately affecting the differentiation of cells (Tzur et al., 2008). Fish oil when added to the diet has been found to upregulate tumor-suppressing miRNAs within intestinal cells, as well as effect steam-cell regulatory pathways through changes in steady level miRNAs (Cui et al., 2017, Davidson et al., 2009). High-fat diets have also been found to increase miR-143 and -145 in mice, the increase in miR143 resulted in increased fasting plasma insulin levels, impaired glucose, and insulin tolerance (Jordan et al., 2011). The nutritional status of pregnant animals is believed to have a tremendous impact on the likelihood of offspring developing metabolic diseases. Adult offspring of female mice fed a high-fat diet before conception showed reduced levels of let-7a, let7b, let7-c, miR-26a, -122, -192, -194, -483, -494-, and -709 during their pregnancy and lactation, in addition to increased expression in the liver of insulin-like growth factor-2 (Igf2), these findings indicate that epigenetic mechanisms may be involved in the maintenance of diet-induced gene expression being maintained into adulthood (Zhang et al. 2009). The let-7 miRNA family regulates glucose metabolism in multiple organs (Frost and Olson, 2011), while miR-122 is a key regulator in cholesterol and fatty acid metabolism (Esau et al., 2006). The long-term impacts of gestational nutrition on offspring and miRNAs within the body still requires more research. Carbohydrates within the diet are a major source of glucose

for many mammals, glucose level within the body has been identified as a factor influencing miRNA expression (Garcia-Segura et al., 2013). Hyperglycemia within mice increased expression of miR21 in the kidneys of type 1 diabetics mice and resulted in decreased expression of renal proteins responsible for suppressing tumors that result in diabetic nephropathy (Dey et al., 2011). On the other end of the spectrum, glucose depletion in mice has been shown to induce the production of miR-466h-5p, which plays a role in regulating myocardial hypertrophy, as a result of oxidative stress and the inhibition of histone deacetylation (Druz et al., 2012). Micronutrients including vitamins, vitamin derivatives, and phytochemicals have also been linked to changes in miRNAs levels (Garcia-Segura et al., 2013, Cui et al., 2017). Continued research into the effects of diet on miRNA expression could result in a better understanding of metabolic diseases and identify miRNA candidates for therapies of these conditions.

Diet has been found to affect the endogenous miRNA profiles of horses. Nulton found in her study horses fed a corn supplemented diet had 37 miRNAs within serum exosomes with different levels of expression after 23 days on the diet. Decreased levels in several members of the *let-7* family (let-7a,c,d, and g), miR-29a, and -143 were found, these miRNAs all relate insulin sensitivity and glucose tolerance (Rottiers and Näär, 2012), MiR-27b was also found to decrease after supplementation and is also decreased during the process of adipogenesis. In this same study three miRNAs linked to adipocyte development (miR 17, -20a, and -20b) and miR-148, associated with increased insulin biosynthesis, were all found to increase after corn supplementation (Nulton,2014). Nulton found miR-20b also increased in horses supplemented with rice bran, in addition to an increase in levels of miR-129a-3p which is associated with prohibiting cell proliferation (Wu et al., 2010). The rice supplemented horses within Nulton's study showed a decrease in miR-33b which has is associated with the control of fatty acid

oxidation and the formation of high-density lipoproteins (Dávalos et al., 2011). To better understand the impact of diet on equine miRNA profiles more studies should be conducted, perhaps with a wider variety of diets given to horses in different life stages and levels of work.

DIGESTION

Horses are considered hindgut fermenters as they are still able to utilize forages like ruminants but do not ferment plant matter until after the ingesta has passed through the length of the small intestine and into the hindgut. The primary sites for microbial fermentation in horses are the cecum and ascending colon. Unlike most monogastric animals, horses have a unique structure to their ascending colon, instead of simply traversing one side of the abdomen, the ascending colon of a horse doubles over on itself in a horseshoe shape and makes up a larger portion of the hindgut than other mammals. The increased volume of both the cecum and ascending colon allows microbes within to ferment forages, breaking the beta 1-4 linkages in cellulose and producing volatile fatty acids (VFAs) that are used within the body for the synthesis of lipids and glucose.

The first step of digestion for animals is the mechanical reduction of food particles through mastication within the oral cavity. Also, within the oral cavity, the initial steps of enzymatic digestion will occur due to salivary enzymes before the food bolus is swallowed and travels through the esophagus to the stomach (Cheeke and Dierenfeld, 2010). The food bolus is moved through the esophagus by waves of muscular contraction called peristalsis. Within the equine stomach mechanical, enzymatic, and chemical digestion will occur to break food particles down further before the ingesta exits the stomach and enters the small intestine. The small intestine, mainly the duodenum, is the primary site of nutrient absorption in mono-gastric animals. Enzymes and bile enter the intestinal lumen through the duodenal papillae and continue

to break down food particles into absorbable compounds. As previously stated horses are hind-gut fermenters and break down the cellulose found within plants through microbial fermentation within the cecum and ascending colon (Ralston, 2007). The transverse and descending colon of the horse mainly functions to absorb water from the ingesta and form fecal balls out of the indigestible portion of the feed.

Carbohydrate digestion and metabolism

Most rations for horses today use a feed concentrate like corn, barley, or a grain mix to provide horses with the energy needed to perform at or above their owner's standards. These feed concentrates contain non-structural carbohydrates (NSC), starches and sugars, and structural carbohydrates, cellulose, and lignin (Ralston, 2007). Mammals do not produce enzymes capable of breaking down beta1,4 linkages, as a result, they have developed means of microbial fermentation to break down these links in structural carbohydrates. As previously mentioned within horses this microbial formation happens within the cecum and large intestine and results in the production of VFAs which can then be utilized by the body. The production of these VFAs can contribute to meeting up to 30% of the horse's energy needs for maintenance (Glinsky et. al, 1976). The VFA produced in the largest amount is acetate which is readily usable by the body as an energy source, propionate and butyrate are also produced through microbial fermentation.

Non-structural carbohydrates such as starch are primarily digested in the foregut of the horse. As food exits the stomach through the pylorus and into the duodenum a small amount of HCl also enters the intestines and triggers the release of pancreatic enzymes, including amylase, into the intestinal lumen which breaks down ingested carbohydrates particles until they have all been converted to glucose or glycogen. Some starches can be classified as resistant starches and pass through the small intestine into the large intestine without being broken down due to the

enzymes being unable to properly attach the starches (Englyst and Englyst, 2005). Glucose is then absorbed through the intestinal wall and into the blood to be distributed throughout the body to cells in need of glucose. Cells put out insulin receptors when glucose is required and upon insulin binding glucose is brought through the cell membrane into the cytosol (Cheeke and Dierenfeld, 2010). A marked increase in insulin production occurs after a meal as a breakdown of the carbohydrates within the meal causes an increase in blood glucose concentration, requiring insulin to push the glucose into cells. Within the cytosol of the cell, glycolysis occurs which converts the glucose into pyruvate that can be utilized by the citric acid cycle and electron transport chain within the mitochondria to produce ATP. Recent studies have found the ingestion of large amounts of carbohydrates can lead to transient hyperinsulinemia in some horses and long-term supplementation of diets high in NSC contribute to increased insulin resistance (de Laat, McGree, and Sillence, 2016, Durnham et al., 2019, Treiber et al., 2006)

Lipid digestion and metabolism

Lipids are an important part of the equine diet as well as integral to the structure of cells as cell membranes are made up of phospholipids. When a lipid is ingested the first step to breaking down the fat involves lipase enzymes emulsifying the triglyceride into free fatty acids and a mono-glyceride, this process starts in the mouth with lingual lipase and continues in the duodenal lumen with pancreatic lipase. Due to the hydrophobic nature of lipids they join and are surrounded by bile salts to form a micelle. The micelles are then absorbed through the intestinal wall into the enterocytes where they have broken down into fatty acids and mono-glycerides again (Cheeke and Dierenfeld, 2010). Within the enterocytes, the lipid by-products will either go through the mono-glycerol acetyltransferase (MAG) pathway or the Phosphatidic Acid Phosphatase (PAP) pathway to reassemble the triglyceride into either a chylomicron or a VLDL

(very low-density lipoprotein). The PAP pathway requires glucose to be converted into glycerol to use within the process of reassembling the triglyceride, unlike the MAG pathway which utilizes the mono-glyceride present within the cell to create triglycerides to put into VLDLs and chylomicrons. Once in the lymphatic system VLDLs and chylomicrons become mature and have the membrane receptors required to bind to high-density lipoproteins (HDL). After binding to HDL, the lipoproteins are transported to adipose tissues with lipoprotein lipase, this results in the fatty acids entering the adipose cell where they are reassembled into triglycerides and stored until use, while the glycerol backbones are transported to the liver and used in gluconeogenesis.

Protein digestion and metabolism

Protein metabolism in horses starts within the stomach where hydrochloric acid and pepsin denature the proteins through hydrolysis starting at the N-terminal. Pepsin is the activated form of pepsinogen which is secreted by chief cells within the mucosal layer of the stomach. As polypeptides enter the small intestine secretin and cholecystokinin are released. The proteases trypsin, chymotrypsin, and carboxypeptidase are also released into the small intestine from the pancreatic duct and further digest the proteins until they are individual amino acids. The amino acids are absorbed by the intestinal epithelial cells and released into circulation (Cheeke and Dierenfeld, 2010). The amino acids travel through the hepatic portal vein where the liver extracts any needed amino acids before the blood is pumped throughout the body to be used in protein synthesis, converted to fat, or used within the citric acid cycle in the form of acetyl CoA.

CONCLUSION

The targets and impacts of miRNAs on various genes continue to be an area of research as we try to understand what roles they play in the everyday functioning of plants and animals,

identify new biomarkers, and investigate their ability to be used as a therapeutic agent. Early miRNA research largely focused on the role miRNAs play in cancers, tumors, and diseases afflicting humans, often using mouse models. Many conditions experienced by humans can be modeled within or are found within companion animals, making research into miRNAs profiles within animals like horses and dogs in addition to the normal animals used in studies like mice and pigs important in the development of information that can be used for translational medicine purposes. To better determine if the uptake of diet-derived miRNAs is truly possible more research needs to be conducted looking at the stability of miRNAs in the digestive tract and the exact method of miRNA transport from the intestinal lumen into circulation, this could also help to create ways of using oral supplementation of encapsulated miRNAs as a therapy for a variety of diseases. Diet is known to have a large impact on the overall health of animals and diets high in NSC have been associated with a higher incidence of insulin resistance within horses (Durnham et al., 2019). miRNAs hold a large potential to act as biomarkers for a variety of diseases and the creation of more economical tests for diagnosis of diseases and early detection of at-risk individuals to allow for appropriate preventive measures to be taken.

The equine industry has seen an increase in obesity as more horses are kept in stalls with lower activity levels, but many still receive some sort of concentrated feed. Many commercial grain mixes fed to horses contain corn, as it is a cheap form of concentrated energy. Corn has a large amount of NSC which has been linked to the development of insulin resistance (Durham et al., 2019). Recent studies within horses have identified miRNAs within horses that could be used as biomarkers for insulin resistance in horses (Santos et al., 2018). Based upon the evidence for diet affecting endogenous miRNA profiles within animals and the ability for diet-derived exogenous miRNAs to be taken up into circulation, we hypothesized that supplementing corn to

horses would result in the uptake of a corn specific miRNA (Zma-mir827-5p) and changes in the profiles of endogenous miRNAs within muscle and serum.

CHAPTER II: IDENTIFICATION OF PLANT MICRORNAS IN EQUINE SERUM AND SKELETAL MUSCLE

INTRODUCTION

Nutrigenomics is the study of the effects of food and its elements on the expression of genes and how the nutritional environment is impacted by genetic variation. This area of science focuses on trying to understand the interaction between specific nutrients and bioactive components of food with genome, proteome, and metabolome to understand how specific nutrients or dietary regimes affect health (Mead, 2007). But despite many studies being done within this area, there is still not a clear understanding of exactly how some elements of food, like miRNAs, impact health. The ability of mammals to uptake exogenous miRNAs from food has been demonstrated (Zhang et al., 2012; Mu et al., 2014, Yang et al., 2015; Yang et al., 2016) with both plant-derived and mammalian derived miRNAs being found within the circulation. miRNAs are small non-coding nucleotide sequences that affect post-transcriptional gene regulation and RNA silencing (Zhang et al. 2012). Plant miRNA specifically affects this process through RNA cleavage and degradation (Taylor et al., 2014).

The ability of mammals to uptake plant-derived miRNAs has been previously shown and suggests that plant miRNAs might be able to play a role in gene regulation within mammals, however, the exact mechanism of transfer and the levels needed within cells to have an impact is still debated (Chan et al., 2017). Some plant miRNAs have been found to have targets within mammals (Zhang et al., 2012, Liu, Y. et. al, 2017), suggesting that diet may have a bigger impact upon animal health than was previously thought. More studies confirming the presence of plant miRNA in mammalian circulation and identification of more target sequences could hold the

possibility of novel treatments and a further understanding of the interaction between diet, insulin resistance, and obesity.

Previous studies looking at dietary miRNAs have mostly been conducted with mice, pigs, and human subjects, with few studies using horses. This study was most interested in if feeding corn would result in the presence of a corn specific miRNA within horses. We hypothesized that plant miRNAs can be found within equine serum and skeletal muscle.

MATERIALS AND METHODS

Animal Care and Feeding Protocols

All experiments were approved by the Colorado State University Institutional Animal Care and Use Committee. Twelve horses were used within this study: two were leased from Colorado State University and ten were already being used for another project being conducted at Colorado State University's Equine Reproduction Lab (Fort Collins, CO). All twelve horses were kept in a dry lot with free-choice water and hay for a 21-day adjustment period before the feeding trial. The horses were all Quarter Horse type mares. Before the start of the 28-day feeding trial, the horses were randomly assigned to either the control group or the corn supplemented diet group. Before starting the feeding trial, all horses were weighed, and body condition scored. The horses were fed to meet the minimum nutritional requirements for maintenance and feeds were analyzed to determine nutritional composition. The horses were fed chopped hay and corn, if in the treatment group, once daily in the morning. The control group received 20 lbs. (9kg) /hd/d of chopped mixed alfalfa and grass hay daily, while the corn supplemented group received 20lbs (9kg) /hd/d of chopped mixed alfalfa and grass hay and 1lb (453 g)/hd/d of steam-flaked corn, these diets were not iso-energetic and both groups had access to round bales. Chemical analysis of feeds was performed to determine nutrient composition

(Appendix Table III). The horses on the corn supplemented diet were gradually acclimated to the diet over 8 days with individual feed bags being utilized to ensure each horse had the correct amount of corn offered to them at each feeding. On days 1-3 horses were fed ¼ lb. (113 g) of corn at each feeding, on days 4 and 5 the horses were moved up to ½ lb. (226g) per feeding, days 6-7 consisted of ¾ lb. (340 g) per feeding. Horses received the full 1 lb. (453 g) of corn from day 8 on.

Serum and Tissues Collection

Serum Collection

10 mL serum separator blood tubes (Becton, Dickinson, and Company (BD), Franklin Lakes, NJ) were used for all blood collections with two 10mL tubes collected at each time point. Blood was collected before the feeding trial began through jugular venipuncture and after 28 days of supplementation at 0, 15, 30, 45, 60, 75, 90, 105, and 120 minutes after eating through intravenous catheters. One sample was also collected 360 minutes after feeding through jugular venipuncture. The intravenous catheters were placed aseptically and secured with a suture at the start of the 120-minute collection period, after each blood collection the lines were flushed with heparinized saline to avoid clotting within the catheter and clamped. Blood samples were then allowed to clot at room temperature for 30 minutes at a 45° angle before centrifugation for 10 minutes at 2,000*g* to separate serum. The serum was separated off and placed into a polypropylene tube and stored at -80° C until RNA isolation.

Muscle Biopsy collection

Before starting (day 0) and after the completion of the feeding trial (day 28), muscle biopsies were taken from the *Gluteus medius* muscle of the horse. Biopsies were taken from the left side of the horse before the feeding trial and from the right side at the end of the trial. The

biopsies were obtained by first shaving the hair off and scrubbing the site with betadine and alcohol before locally blocking the area through subcutaneous administration of lidocaine around the biopsy area. Once blocked using an aseptic technique a biopsy needle was inserted midway between the tuber coxae and ischiatic tuberosity to a depth of 6cm to obtain the tissue sample. Biopsy samples were placed into sterile cryotubes, then snap-frozen in liquid nitrogen before being stored at -80°C until RNA isolation.

RNA Isolation from Serum

RNA was isolated from equine serum using the manufacturer's protocols for TRI Reagent® BD (Molecular Research Center, Inc. Cincinnati, OH). 500µl of serum was added to a tube along with 10µl Polyacryl carrier and 750 µl of TRI Reagent® BD. After samples were lysed using the TRI Reagent® BD, 200µL of chloroform was used to separate the RNA, DNA, and protein portions of the sample and allowed to sit at room temperature for 15 minutes before centrifuging the samples for ten minutes at 12,000*g* at 4ºC. The RNA portion was then transferred to a new tube and precipitated with isopropanol and a Sodium acetate salt solution (pH 5.4). After RNA precipitation had occurred samples were centrifuged for 10 minutes at 12,000*g* and the supernatant was pipetted off. The pellet was then washed with 1 mL of 75% ethanol. This washing procedure was repeated two more times, for a total of three ethanol washes per sample. The sample was then tested on a Nanodrop® Spectrophotometer ND-2000 (Thermo Scientific, Wilmington, DE) to determine RNA concentration. If samples had a concentration higher than 150 ng/ µL of RNA they were treated with DNA-free DNase Treatment and Removal Reagent (Invitrogen/Life Sciences, Grand Island, NY) to remove genomic DNA then RNA purity was re-assessed. If RNA concentrations were below 150ng/µL then they were not subjected to the DNase treatment to ensure enough RNA was present for

adequate amounts of cDNA to be generated later. Samples were then stored at -80°c until the generation of cDNA through reverse transcription occurred.

RNA isolation from Tissue

RNA isolation from skeletal muscle was accomplished through grinding the samples with a mortar and pestle that had been previously washed with ethanol and nanopure water to ensure all contamination was removed. The mortar and pestle were then conditioned with liquid nitrogen and the muscle sample was placed into liquid nitrogen within the mortar and ground into a fine powder. The powder was then transferred to a clean tube and Trizol (Thermo Scientific, Wilmington, DE) was added according to the manufacturer's protocols to lyse the cells within the sample. After lysing the sample 200µL of chloroform was used to separate the RNA, DNA, and protein portions of the sample and allowed to sit at room temperature for 15 minutes before centrifuging the samples for ten minutes at 12,000*g* at 4ºC. The RNA supernatant portion was then transferred to a new tube and precipitated with isopropanol. After RNA precipitation had occurred samples were centrifuged for 10 minutes at 12,000*g* and the supernatant was pipetted off. The pellet was then washed with 1 mL of 75% ethanol. This washing procedure was repeated two more times, for a total of three ethanol washes per sample. The sample was then tested on a Nanodrop® Spectrophotometer ND-2000 (Thermo Scientific, Wilmington, DE) to determine RNA concentration the samples were treated with a DNA-free DNase treatment and removal reagent to remove any genomic DNA, then RNA purity and concentration were re-examined. Samples were then stored at -80°c until the generation of cDNA through reverse transcription occurred.

Plant RNA Isolation

RNA was isolated from the hay and corn samples using TRI reagent and the protocols produced by the manufacturer with some modifications. After the sample was homogenized using a mortar and pestle, previously cleaned with ethanol and nanopore water, and allowed to dry, it was combined with 750 µL of TRI reagent in a 1.75-ml tube and homogenized. Any extracellular material was removed through centrifugation at 12,000*g* for 10 minutes and the supernatant was transferred to a new tube for further preparation. As mentioned above, the RNA phase was separated using 200 µL chloroform. The RNA precipitation was conducted through the addition of 250 µL isopropanol and 25 µL salt precipitation solution. After precipitation occurred the sample was centrifuged for 10 minutes at 12,000*g* and washed with 75% ethanol, this step was repeated two more times. The sample was treated with a DNA-free DNase treatment and removal reagent to remove any genomic DNA and RNA purity assessed with a Nanodrop® Spectrophotometer ND-2000 (Santos et al., 2018). Samples were then stored at -80°c until the generation of cDNA.

Reverse Transcription

cDNA was generated using the miScriptII RT kit (Qiagen Valencia, CA). 4µL 5x HiSpec Buffer and 2µL 10x Nucleics mix was used for the master mix for each reaction. Each tube contained 6µl of the master mix, enough RNA for 1000ng per reaction, and nuclease-free water, the total volume of each tube was 18µl. After mixing the reaction 2µL of Reverse transcriptase mix was added to the tube and the tube placed in a thermocycler. The reaction was incubated for 60 minutes at 37°C, then at 95°C for 5 minutes. The cDNA was then diluted with 100µL of nanopure water for a total volume of 120 µL and a cDNA concentration of 500 pg/µL. The diluted cDNA was stored at -20ºC until further use in real-time PCR.

Real-Time PCR Analysis

Three mature plant miRNAs were selected for analysis due to previous reports of their expression in serum, or expression in the specific plant material feed (Xue et al., 2008; Zhang et al., 2009; Jiao et al., 2011; Zhang et al., 2012, Nulton, 2014) (Appendix table II). The first plant miRNA used was Ath-miR156a which was found to be conserved across alfalfa and corn and identified within equine tissue and serum previously, Zma-miR827-5p was found to only be in corn feed, and Osa-mir1866-3p was found to only be in rice (Nulton, 2014). The relative level of the chosen three mature plant miRNAs was assessed in total equine serum and skeletal muscle. Each real-time PCR reaction contained 6 µL of total reaction, including 3µL 2x QuantiTect SYBR Green PCR Master Mix, .60µL 10x miScript Universal reverse primer,1.15 µl of nuclease-free water, 0.25 µL of cDNA, and 1µl miRNA specific forward primer. Cycle conditions consisted of reaction initiation at 95°C for 15 minutes, followed by 45 cycles of 94°C for 15 seconds to denature, 55°C for 30 seconds to anneal, and 70°C for 30 seconds for extension. Plates were run in duplicate and miRNAs were considered present if they were present at cycle number <37 and confirmed with amplification curves and singular melt peaks (Santos et al., 2018). A negative RT control, non-template control, and positive RNU1 control were included on all plates.

Statistical Analysis

To determine if plant and corn specific miRNAs are present within equine serum and muscle, raw Ct values were normalized to RNU1 (appendix table II), a small RNA used as a reference gene within human serum studies (Sanders et. al, 2012) and had a standard deviation of less than 2.5 for all samples. This normalized value was considered ΔCt. The 1/ΔCt values were

not normally distributed, so data were analyzed using the Kruskal-Wallis test and Dunn's test in R version 3.5 statistical software with $P \leq 0.05$ being considered significant.

RESULTS

Horses were weighed and body condition scored on days 0 and day 28. No time, treatment, or time by treatment effect was found (Table 1).

Analysis of samples from corn and hay fed by QuantiTect qRT-PCR revealed that ath-miR156a was present in all feeds. The rice miRNA (osa-miR1866-3p) was only detectable in the corn feed and the corn miRNA (zma-miR827-5p) as expected was only present in the corn feed (Table 2).

Plant miRNAs (ath-miR156a, zma-miR827-5p, and osa-miR1866-3p) were detectable in total serum miRNA on day 0 and day 28. Ath-miR156a was found in all horses at every time no treatment, time, or time by treatment effect was found (figure 5). Zma-miR827-5p was detectable in horses in both groups with a treatment effect ($P<.05$) present and horses fed corn having overall higher serum levels of this miRNA, no time or time by treatment effect were found (Figure 6). Osa-miR1866-3p was found in all horses at every time, no treatment, time, or time by treatment effect existed (Figure 7).

All three plant miRNAs (ath-miR156a, zma-miR827-5p, and osa-miR1866-3p) were detected in muscle samples within each group (figures 8,9, and 10). No treatment, time, or time by treatment effects were found for any of the plant miRNAs tested.

Table 1: Horse Body Condition Score and Weight. Horses were weighed on a livestock scale on day 0 and day 28 of the trial. All horses were body condition scored using the Henneke system on a scale of 1-9. Shown as group mean and SD

Group	Body Condition Score	Bodyweight (kg)
Corn Day 0	6.17±0.41	553.91±26.90
Control Day 0	6.00±0.58	552.78±35.84
Corn Day 28	6.17±0.61	582.11±43.50
Control Day 28	6.08±0.74	567.07±33.07

Table 2: Plant miRNA Expression Across Feeds. Levels of plant miRNAs were detected using qRT-PCR in the round bales, chopped hay, and corn fed to horses throughout the feed trial.

miRNA	RNA sample	Ct
Ath-miR156a	Mixed Grass Hay Round Bale	33.3
Ath-miR156a	Chopped Mixed Grass Hay	34
Ath-miR156a	Steam Flaked Corn	26.3
Zma-miR827-5p	Mixed Grass Hay Round Bale	Undetectable
Zma-miR827-5p	Chopped Mixed Grass Hay	Undetectable
Zma-miR827-5p	Steam Flaked Corn	23.4
Osa-mir1866-3P	Mixed Grass Hay Round Bale	Undetectable
Osa-mir1866-3P	Chopped Mixed Grass Hay	Undetectable
Osa-mir1866-3P	Steam Flaked Corn	36

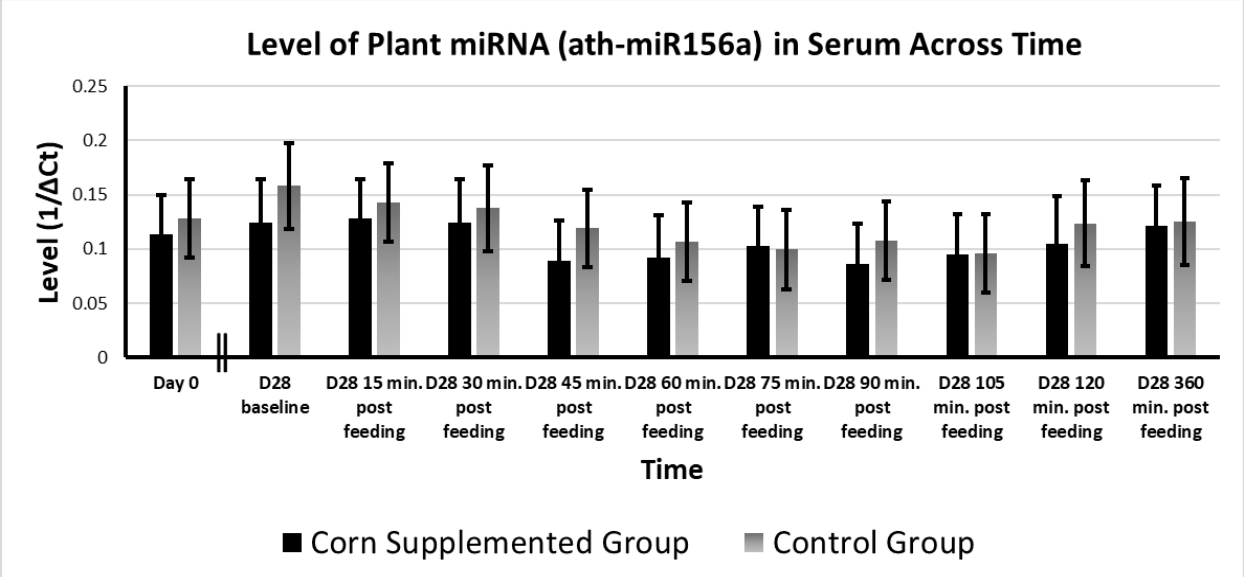

Figure 5: Level of Plant miRNA (ath-miR156a) in Serum Across Time. qRT-PCR was performed to determine levels of ath-miR156a in serum across all diets at day 0 and day 28. No treatment, time, or time by treatment effect was found. Shown as group means with 95% CI. Data were normalized to RNU1.

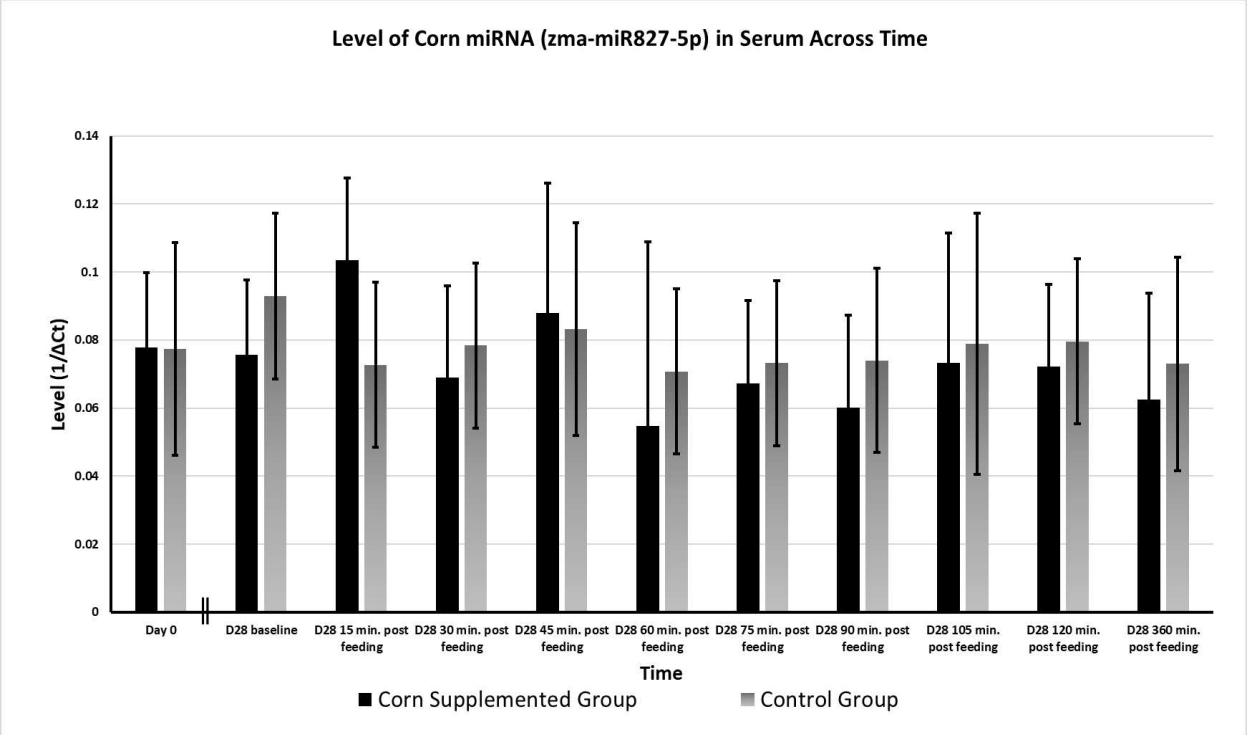

Figure 6: Level of Corn miRNA (zma-miR827-5p) in Serum Across Time. qRT-PCR was performed to determine levels of zma-mir 827-5p in serum across all diets at day 0 and day 28. A treatment effect for the corn group was found (P<.05). No time or time by treatment effect was found. Shown as group means with 95% CI. Data were normalized to RNU1.

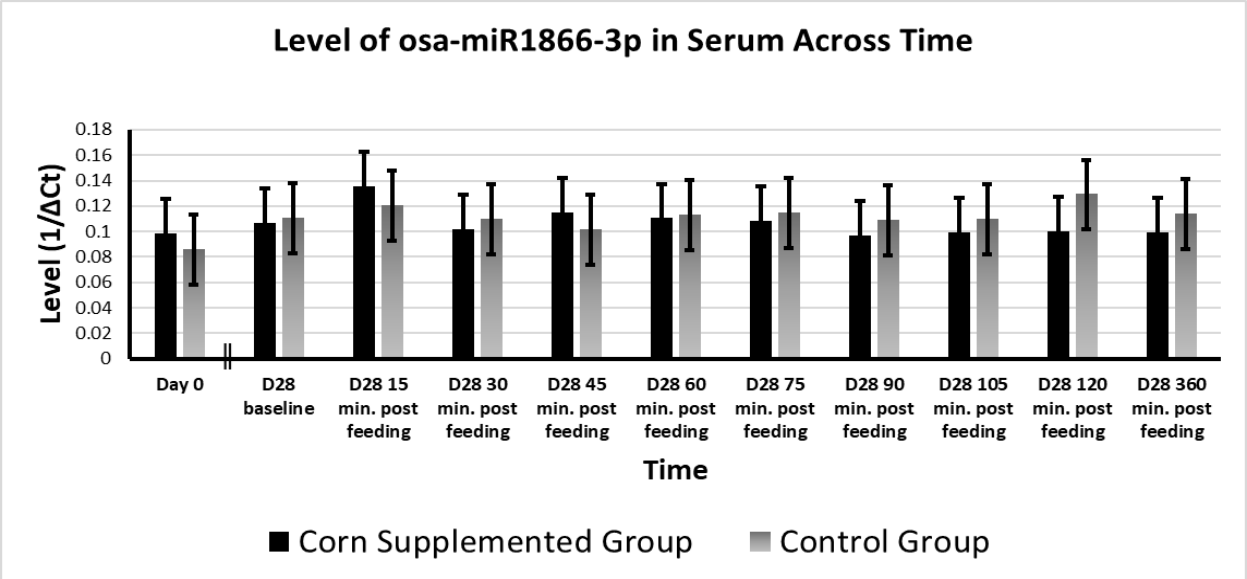

Figure 7: Level of osa-miR1866-3p in Serum. qRT-PCR was performed to determine levels of osa-mir1866-3p in serum across all diets at day 0 and day 28. No treatment, time, or time by treatment effect was found. Shown as group means with 95% CI. Data were normalized to RNU1.

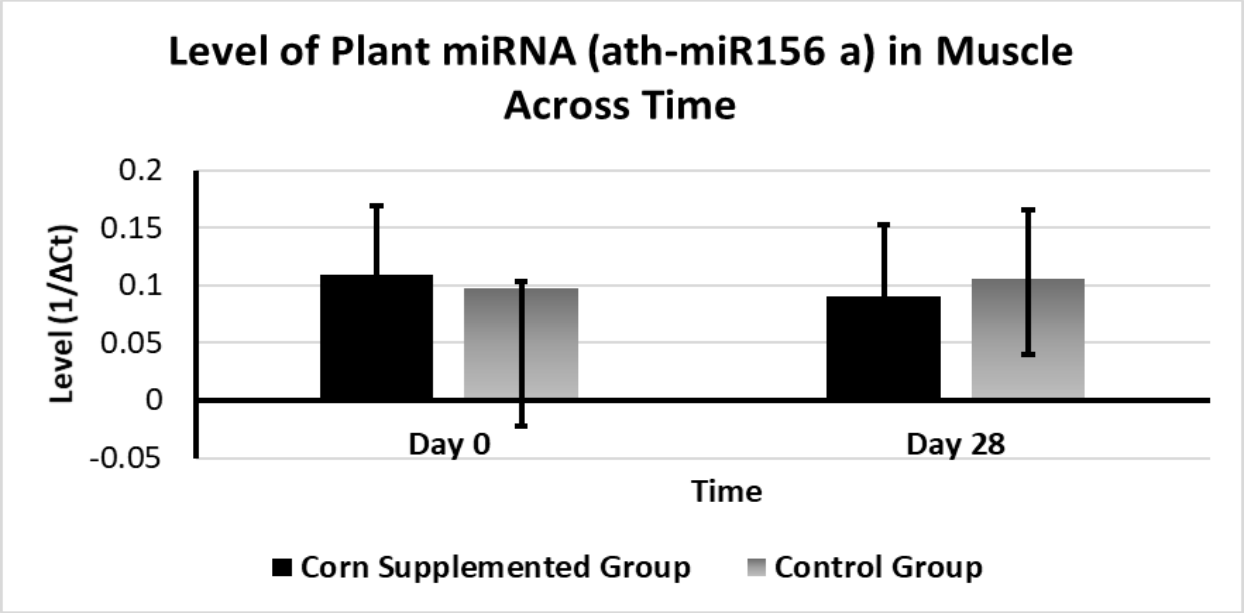

Figure 8: Relative Level of Plant miRNA (ath-miR156a) in Muscle Across Time. qRT-PCR was performed to determine levels of ath-miR156a in muscle across all diets at day 0 and day 28. No treatment, time, or time by treatment effect was found Shown as group means with 95% CI. Data were normalized to RNU1.

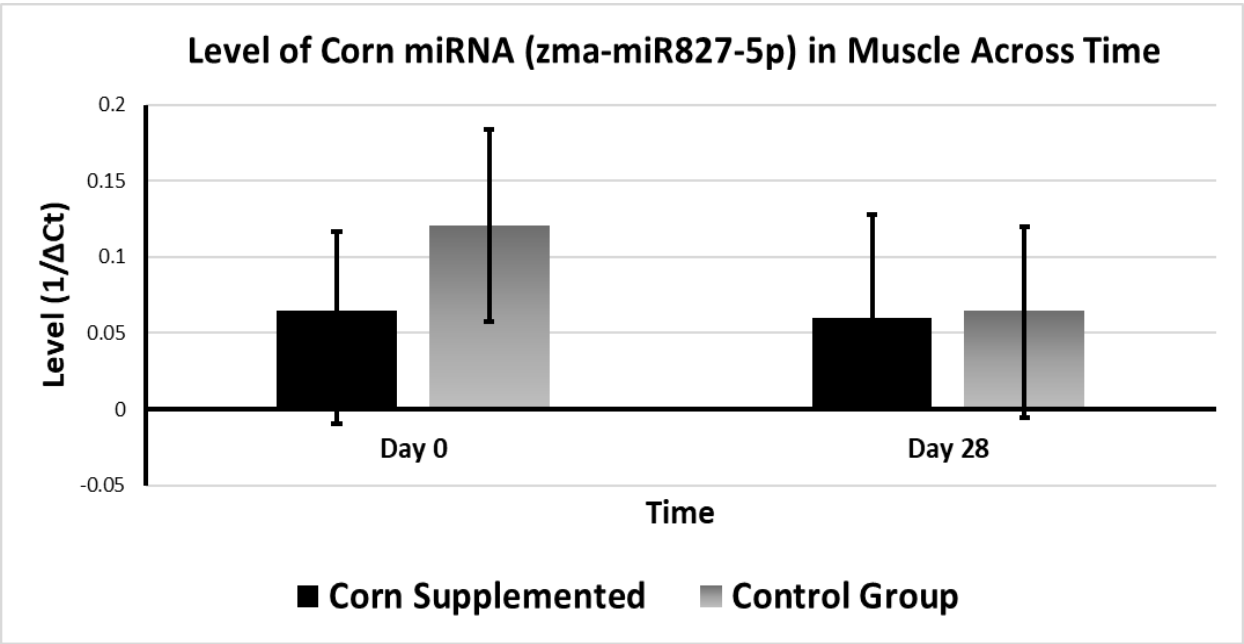

Figure 9: Level of Corn miRNA (zma-mir 827-5p) in Muscle Across time. qRT-PCR was performed to determine levels of zma-miR827- 5p in muscle across all diets at day 0 and day 28. No treatment, time, or time by treatment effect was found. shown as group means with 95% CI. Data were normalized to RNU1.

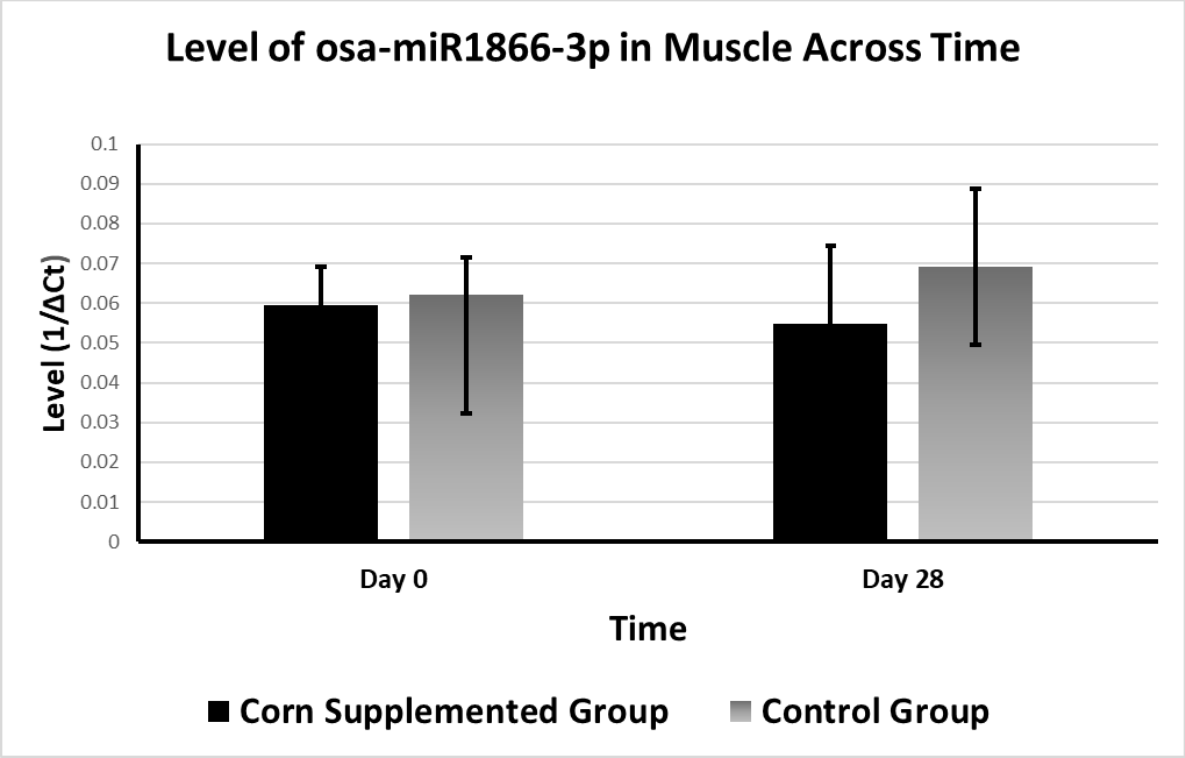

Figure 10: Level of osa-miR1866-3p in Muscle. qRT-PCR was performed to determine levels of osa-miR1866-3p in muscle across all diets at day 0 and day 28. No treatment, time, or time by treatment effect was found. Shown as group means with 95% CI. Data were normalized to RNU1.

DISCUSSION

To determine if plant miRNAs can be absorbed from feedstuffs into serum, samples were collected on Day 0 and Day 28 of a feeding trial. Serum on day 28 was collected before feeding, 15, 30, 45, 60, 75, 90, 105, 120 minutes, and 360 minutes after feeding corn to the corn group (CORN). The control group (CONT) was not fed hay at the start of the collection period on day 28 but it is unknown when the last time the horses ate as they had access to hay in their pens before removing them for sample collections. The presence of plant miRNAs (ath-miR156a, zma-miR827-5p, osa-miR866-3p) in total serum samples is similar to the results from previous studies on mice, humans, and calves (Zhang et.al, 2012, Wang et al, 2012), while different from others that did not find plant miRNAs in total serum within mice (Dickinson et al., 2013) and

horses (Nulton 2014). In Nulton's study, the only plant miRNA identified in equine serum was ath-miR156a and it was only found in exosomes (Nulton, 2014). The body of research that exists looking at the uptake of dietary miRNAs into circulation shows many are inconsistently picked up or detected in individual animals (Yang et. al, 2015), the wide variation in detectability of plant miRNAs within circulation could be due to re-uptake of the miRNAs into tissues, extremely low levels undetectable within cycle cutoffs, or individual variations in the absorption of intestinal contents, but further research needs to be conducted to better understand the process of dietary miRNA uptake and could illuminate the inconsistently present in current studies. The results revealed that ath-miR156a and osa-miR18663p were both detectable in total serum of all horses with no difference between treatment or time and no time by treatment effect existed. Although the corn miRNA (zma-miR827-5p) was present in both groups before corn was supplemented to horses a treatment effect was found where the level within the serum of CORN was higher than CONT ($P<.05$). The reason for the corn and rice miRNAs being present before supplementation is not clear as all horses were on a forage only diet for 14 days before taking day 0 samples and to our knowledge did not receive anything other than forage during this adjustment period; However, the horses were on the same premises and had intermittent access to the same pen system as horses receiving grain containing corn and rice bran for a different study looking at the effects of nutrition on oocytes (Catandi et al., 2020). The mares used within our study were also being teased and palpated up to two times a week for an unrelated study after palpation had been done in the stocks the mares were let out into a large pen that had access to the pens used by Catandi et al. to feed their horses if any feed had been left on the ground horses from our study could have eaten them. Catandi et al.'s study started before and extended beyond the end of our study. The horses were kept in an open-air pen and high winds do occur in the

geographical area where the study occurred, so although the corn was contained in feed bags and the horses were taken out of the community pen when being fed corn flakes could have been blown into the pen unnoticed and consumed.

The corn miRNA (zma-miR827-5p) had the most variation in detection within the horses in total serum in this study, as horses did not have it appear in every time point, and the horses for which the miRNA was undetectable varied with time. Levels of this miRNA spiked 15 minutes after ingestion of corn (figure 6), the earliest point at which ingested food can exit the equine stomach and enter the duodenum for further digestion and absorption of nutrients. This spike was not found to be statistically significant but suggests that miRNAs are taken into circulation within the early portion of the digestive tract, like the small intestine. The disappearance and reappearance of the miRNA could be the result of tissue uptake and then later release back into the circulatory system, however the exact details of miRNA uptake and release into circulation are not completely known. A study utilizing labeled miRNAs could help to provide more clarity on why miRNA concentrations in circulation vary with time. Within our study horses had access to water throughout the whole sample collection process, the intake of water holds the potential to dilute miRNA concentrations within serum and cause fluctuations in detectable levels as water is absorbed by the body. Another reason for the appearance and disappearance of the miRNA in total serum maybe this specific miRNA may be more highly expressed within exosomes in serum, which were not tested in this particular study, or has a higher rate of degradation within the GI system of horses than the other two plant-based miRNAs tested. Zma-miR827-5p was selected to be used based upon previous studies showing it was expressed in corn (Zhang et. al. 2009, Jiao et. al, 2011) and that it was detectable in corn

despite cooking method, although steaming the corn did result in a large decrease in levels of this miRNA in another study (Lou et. al, 2017).

All plant miRNAs (plant, corn, and rice) tested were detectable in equine muscle at Day 0 and Day 28, no treatment, time, or time by treatment effect existed. The presence of plant miRNAs in tissue is in line with other studies that have found plant miRNAs to be taken up into mammalian tissues (Lou et. al, 2017, Zhang et. al 2012). Unlike Nulton's study which only found ath-miR156a present in equine tissues regardless of diet (Nulton,2014), our study also identified zma-miR827-5p and osa-miR18663p in equine tissue in addition to ath-miR156a. As was previously mentioned another feeding study was being conducted at the same time as our study with some shared facilities, with groups being fed grain containing corn and rice bran, creating a potential source of contamination despite horses having very minimal access to facilities where feed could have been present for the horses within our study to consume (Catandi et al., 2020). As the effect of feeding corn on miRNA presence in horses was the main focus of this study we had a special interest in the potential presence of corn miRNA in muscle. As previously stated no treatment effect was found for the corn specific miRNA (zma-miR827-5p) within muscle, this may indicate that a larger amount of corn should be fed to horses if future studies are conducted in this area and specific care should be taken to avoid environmental contamination should always be included. Currently, there is no information on the exact mechanism of how exogenous miRNAs within circulation become incorporated into cells within the body. This study did not take samples from smooth muscle cells within the GI organs, the proximity of these cells to the intestinal lumen creates the possibility that these cells could have higher levels of plant-derived miRNAs within them and may show a notable difference sooner and with lower levels of supplementation compared to skeletal muscles. Although zma-miR827-

5p was found in both groups on Day 0 the corn supplemented group maintained roughly the same level on Day 28, while the control group (hay only diet) had a reduction in the relative level from Day 0 to Day 28 (Figure 9).

The presence of osa-miR1866-3p a rice miRNA (Nulton, 2014, Xue et.al, 2008.) in the corn feed sample could be a result of where the corn was obtained from, the corn used within the study was obtained from a pile stored in a covered open-air shed with Brewer's grains in the next bay over, which can contain rice, the area where this shed is can get windy at times and the same front loader is used on all feed piles to load the feed truck. Osa-miR1866-3p, a rice specific miRNA, had a Ct value of 36, just barely inside of the cutoff window of <37 cycles that were used within this study, indicating very little was present within the corn feed. This however does not explain why the rice specific miRNA was detected in samples from both groups before and after the feeding trial, this particular miRNA did not have any treatment effects for muscle.

The unexpected detection of corn and rice miRNAs (zma-miR827-5p and osa-miR1866-3p, respectively) in both muscle and serum before supplementation could also be caused by the primer binding to an equine miRNA of a similar sequence, although the primers were created specifically for each plant miRNA, an oxidizing agent was not added to any of the samples. Plant miRNAs are structurally different than mammalian miRNAs as they contain a 2'-O-Me modification on the 3' end which makes them resistant to oxidation. Due to their resistance to oxidation, an oxidizing agent like periodate can help to degrade mammalian miRNAs, but it will also degrade immature plant miRNAs as they do not have the same modifications as mature miRNAs (Huang et al., 2017). Previous studies looking for the uptake of dietary miRNAs also did not treat samples with periodate and subsequently, some plant miRNAs that have been identified in human plasma have been found to match human miRNAs (Witwer, 2018). We did

conduct a BLAST search on zma-miR827-5p and did not find any equine sequences that matched this miRNA sequence, however Lou et al. did a study where they feed fresh corn to pigs, and zma-miR827-5p was detected in serum and muscle before the addition of an oxidizing agent after which it disappeared suggesting that it may not have been a true plant miRNA or a non-mature variant that was detected (Lou et.al, 2017). The possibility of primer mismatch cannot be entirely ruled out, although the guidelines for annealing temperatures were followed and the primer sequences were taken from Nulton's study which was also conducted on horses (Nulton, 2014). The continued debate over the uptake of dietary miRNA likely will continue until a gold standard method is adopted by all researchers to ensure identified miRNAs are true plant miRNAs.

Our study did not investigate the potential interaction between plant miRNA and mammalian genes. Currently, the corn miRNA (zma-miR827-5p) and rice miRNA (osa-miR1866-3p) used within our study do not have any predicted mammalian target genes identified. A previous study identified four potential mammalian gene targets for ath-miR156a; ALG2, SUCLG2, HIF3A, and F11R (Nulton, 2014). ALG2 is a member of the glycosyltransferase 1 family, glycosyltransferases are involved in the biosynthesis of polysaccharides, disaccharides, and monosaccharides, this gene is predicted to be repressed by ath-miR156a along with HIF3A, a gene related to hypoxia. Ath-miR156a is predicted to cleave SUCLG2, a succinyl-COA ligase, and F11r, an adhesion protein (Nulton, 2014). In future studies predicted mammalian gene targets for the three plant miRNAs used within our study should be further explored to see if diet-derived miRNAs play a role in preventing or causing disease within hosts.

Our study shows that plant-derived miRNAs can be detected in equine serum and tissue after ingestion, but more studies within horses should be conducted to confirm these findings as the majority of studies looking at this phenomenon have been conducted in mice, pigs, and humans which are physiologically different than horses making comparisons between studies hard. The specific plant miRNAs used in this study are not heavily used by other research studies in this area, so direct comparison is not possible for many studies, however, the ability to uptake diet-derived miRNA likely is not impacted by the individual miRNA itself. Continued research into the mechanism by which diet-derived miRNAs exit the intestinal lumen and into circulation, either as free miRNAs or encapsulated in microvesicles such as exosomes could help to resolve the current debate within the scientific community. The ability of plant miRNAs to be absorbed into circulation and tissues and determining the levels required per cell to have an impact on mammalian gene regulation holds the potential for new therapeutic or diagnostic techniques to be developed within horses and other mammals.

CHAPTER III: THE EFFECT OF CORN SUPPLEMENTATION ON THE ENDOGENOUS MIRCORNA PROFILE IN EQUINE SERUM AND SKELETAL MUSCLE

INTRODUCTION

The equine digestive system is evidence that through the course of evolution horses met their nutritional requirements through grazing, usually up to 16 hours a day. But due to the large role domesticated horses played as draft animals and the primary mode of transportation the diets of horses began to contain more grain to provide the animals with a concentrated source of energy to ensure they met the needs of their owners. Although not the primary source of transportation anymore, many horses today are fed some sort of grain concentrate. The variety of concentrate feeds that are available today is constantly growing, many of these feeds contain a concentrated form of energy in the form of cereal grains, like corn. Corn has a high energy concentration due to having a high non-structural carbohydrate (NSC) content, composed of starch and simple sugars. The combination of horses evolving to survive harsh winters and plentiful summers through the selection of thrifty genes, diets containing high levels of NSC, and many horses being confined to stalls with inadequate exercise has resulted in an increase of obesity within the equine population, often associated with insulin resistance and intolerance to glucose (Johnson et. al, 2012). Rapid ingestion of a large amount of NSC, like in the case of a horse getting into a grain bin, has also been associated with the onset of laminitis (Eades et. al, 2014). The relationship between diet and metabolic disease is still not completely understood in many mammals and new studies are constantly being conducted.

Diet has been shown to alter miRNA in previous studies with amino acids, carbohydrates, and fatty acids being linked to changes in miRNA expression (García-Segura et al, 2013). As

incidences of equine obesity and metabolic diseases increase the role of diet on the body is becoming increasingly important. If diets can be designed to change miRNA profiles to prevent or treat a disease within mammals a whole new emphasis on the therapeutic uses of nutrition could develop. The goal of this study is to compare levels of 277 endogenous equine miRNAs within horses on a forage only diet (CONT) to horses on a diet that includes corn supplementation (CORN). We hypothesize that feeding corn will result in a different profile of endogenous miRNAs within equine serum and skeletal muscle compared to horses on a forage only diet.

MATERIALS AND METHODS

Animal care and feeding protocols

All experiments were approved by the Colorado State University Institutional Animal Care and Use Committee. Twelve horses were used within this study: two were leased from Colorado State University and ten were already being used for another project being conducted at Colorado State University's Equine Reproduction Lab (Fort Collins, CO). All twelve horses were kept in a dry lot with free-choice water and hay for a 21-day adjustment period before the feeding trial. The horses were all Quarter Horse type mares. Before the start of the 28-day feeding trial, the horses were randomly assigned to either the control group or the corn supplemented diet group. Before starting the feeding trial, all horses were weighed, and body condition scored. The horses were fed to meet the minimum nutritional requirements for maintenance and feeds analyzed to determine nutritional composition. The horses were fed chopped hay and corn, if in the treatment group, once daily in the morning. The control group received 20 lbs. (9kg) /hd/d of chopped mixed alfalfa and grass hay daily, while the corn supplemented group received 20lbs (9kg) /hd/d of chopped mixed alfalfa and grass hay and 1lb

(453 g)/hd/d of steam-flaked corn, these diets were not iso-energetic and both groups had access to round bales. Chemical analysis of feeds was performed to determine nutrient composition (Appendix Table III). The horses on the corn supplemented diet were gradually acclimated to the diet over 8 days with individual feed bags being utilized to ensure each horse had the correct amount of corn offered to them at each feeding. On days 1-3 horses were fed ¼ lb. (113 g) of corn at each feeding, on days 4 and 5 the horses were moved up to ½ lb. (226g) per feeding, days 6-7 consisted of ¾ lb. (340 g) per feeding. Horses received the full 1 lb. (453 g) of corn from day 8 on.

Serum and Tissue Collection

Serum Collection

10 mL serum separator blood tubes (Becton, Dickinson, and Company (BD), Franklin Lakes, NJ) were used for all blood collections with two 10mL tubes collected at each time point. Blood was collected before the feeding trial began through jugular venipuncture and after 28 days of supplementation at 0, 15, 30, 45, 60, 75, 90, 105, and 120 minutes after eating through intravenous catheters. One sample was also collected 360 minutes after feeding through jugular venipuncture. The intravenous catheters were placed aseptically and secured with a suture at the start of the 120-minute collection period, after each blood collection the lines were flushed with heparinized saline to avoid clotting within the catheter and clamped. Blood samples were then allowed to clot at room temperature for 30 minutes at a 45° angle before centrifugation for 10 minutes at 2,000*g* to separate serum. The serum was separated off and placed into a polypropylene tube and stored at -80° C until RNA isolation.

Muscle Biopsy collection

Before starting (day 0) and after the completion of the feeding trial (day 28), muscle biopsies were taken from the *Gluteus medius* muscle of the horse. Biopsies were taken from the left side of the horse before the feeding trial and from the right side at the end of the trial. The biopsies were obtained by first shaving the hair off and scrubbing the site with betadine and alcohol before locally blocking the area through subcutaneous administration of lidocaine around the biopsy area. Once blocked using an aseptic technique a biopsy needle was inserted midway between the tuber coxae and ischiatic tuberosity to a depth of 6cm to obtain the tissue sample. Biopsy samples were placed into sterile cryotubes, then snap-frozen in liquid nitrogen before being stored at -80°C until RNA isolation.

RNA Isolation from Serum

RNA was isolated from equine serum using the manufacturer's protocols for TRI Reagent® BD (Molecular Research Center, Inc. Cincinnati, OH). 500µl of serum was added to a tube along with 10µl Polyacryl carrier and 750 µl of TRI Reagent® BD. After samples were lysed using the TRI Reagent® BD, 200µL of chloroform was used to separate the RNA, DNA, and protein portions of the sample and allowed to sit at room temperature for 15 minutes before centrifuging the samples for ten minutes at 12,000*g* at 4ºC. The RNA portion was then transferred to a new tube and precipitated with isopropanol and a Sodium acetate salt solution (pH 5.4). After RNA precipitation had occurred samples were centrifuged for 10 minutes at 12,000*g* and the supernatant was pipetted off. The pellet was then washed with 1 mL of 75% ethanol. This washing procedure was repeated two more times, for a total of three ethanol washes per sample. The sample was then tested on a Nanodrop® Spectrophotometer ND-2000 (Thermo Scientific, Wilmington, DE) to determine RNA concentration. If samples had a

concentration higher than 150 ng/ µL of RNA they were treated with DNA-free DNase Treatment and Removal Reagent (Invitrogen/Life Sciences, Grand Island, NY) to remove genomic DNA then RNA purity was re-assessed. If RNA concentrations were below 150ng/µL then they were not subjected to the DNase treatment to ensure enough RNA was present for adequate amounts of cDNA to be generated later. Samples were then stored at -80°c until the generation of cDNA through reverse transcription occurred.

RNA isolation from Tissue

RNA isolation from skeletal muscle was accomplished through grinding the samples with a mortar and pestle that had been previously washed with ethanol and nanopure water to ensure all contamination was removed. The mortar and pestle were then conditioned with liquid nitrogen and the muscle sample placed into liquid nitrogen within the mortar and ground into a fine powder. The powder was then transferred to a clean tube and Trizol (Thermo Scientific, Wilmington, DE) was added according to the manufacturer's protocols to lyse the cells within the sample. After lysing the sample 200µL of chloroform was used to separate the RNA, DNA, and protein portions of the sample and allowed to sit at room temperature for 15 minutes before centrifuging the samples for ten minutes at 12,000g at 4ºC. The RNA supernatant portion was then transferred to a new tube and precipitated with isopropanol. After RNA precipitation had occurred samples were centrifuged for 10 minutes at 12,000g and the supernatant was pipetted off. The pellet was then washed with 1 mL of 75% ethanol. This washing procedure was repeated two more times, for a total of three ethanol washes per sample. The sample was then tested on a Nanodrop® Spectrophotometer ND-2000 (Thermo Scientific, Wilmington, DE) to determine RNA concentration the samples were treated with a DNA-free DNase treatment and removal reagent to remove any genomic DNA, then RNA purity and concentration were re-

examined. Samples were then stored at -80°c until the generation of cDNA through reverse transcription occurred.

Reverse Transcription

cDNA was generated using the miScriptII RT kit (QIAGEN). 4µL 5x HiSpec Buffer and 2µL 10x Nucleics mix was used for the master mix for each reaction. Each tube contained 6µl of the master mix, enough RNA for 1000ng per reaction, and nuclease-free water, the total volume of each tube was 18µl. After mixing the reaction 2µL of Reverse transcriptase mix was added to the tube and the tube placed in a thermocycler. The reaction was incubated for 60 minutes at 37°C, then at 95°C for 5 minutes. The cDNA was then diluted with 100µL of nanopure water for a total volume of 120 µL and a cDNA concentration of 500 pg/µL. The diluted cDNA was stored at -20ºC until further use in real-time PCR.

Real-Time PCR Analysis

qRT-PCR was conducted using 277 endogenous equine miRNA primers (Appendix table I) previously used within this lab for other equine studies. qRT-PCR was performed on all muscle samples, day 0 serum, day 28 serum 1 and 6 hours after feeding, the choice to not test all serum samples was based on available supplies, funding, and time. Each real-time PCR reaction contained 6 µL of total reaction, including 3µL 2x QuantiTect SYBR Green PCR Master Mix, .60µL 10x miScript Universal reverse primer,1.28 µl of nuclease-free water, 0.12 µL of cDNA, and 1µl miRNA specific forward primer. Cycle conditions consisted of reaction initiation at 95°C for 15 minutes, followed by 45 cycles of 94°C for 15 seconds to denature, 55°C for 30 seconds to anneal, and 70°C for 30 seconds for extension. Plates will be run in duplicate and miRNAs will be considered present if they are present at cycle number <37 and confirmed with

amplification curves and singular melt peaks (Santos et al., 2018). A negative RT control, non-template control, and positive RNU1 control were included on all plates.

Statistical Analysis

To determine the change in equine miRNA levels within serum between day 0 and day 28 of the dietary treatments, raw Ct values were normalized to the geometric mean of 3 endogenous controls (eca-mir376c, eca-mir14030, and ecamir770) that all had a standard deviation of less than 1.1 for all serum samples. The raw Ct values from the muscle samples were also normalized to the mean of 3 endogenous controls (eca-mir 15a, eca-mir6155p, eca-mir 770) which had standard deviations of less than 2 amongst all muscle sample. Only miRNAs detected within all samples at all time points were used, 41 miRNAs in serum and 16 miRNAs in skeletal muscle fit these criteria and were used for statistical analysis. After normalizing all data this value was identified as ΔCt, the ΔCt for each gene on Day 0 was then used as the calibrator for calculating ΔΔCt (ΔΔCt=ΔCt-calibrator) allowing each horse to act as its control. ΔΔCt was then used for further statistical analysis. Data were analyzed using a mixed model within the SAS statistical software, with $P \leq 0.05$ being considered significant.

RESULTS

As stated in chapter II horses were weighed and body condition scored on days 0 and day 28. No time, treatment, or time by treatment effect was found (Table 1).

Analysis of equine serum found 12 of the 41 miRNAs chosen for statistical analysis, due to appearing in all horses at every time point, to have a significant treatment effect ($P<.05$) (figure 11 and table 3). 11 of the 12 miRNAs resulted in a significant downregulation of the gene within the CORN group compared to Day 0, while ecamir4903p had a significant upregulation within the CONT group compared to Day 0 ($P<.05$) (Table 3). Analysis of eca-mir 129a5p

showed a time by treatment effect for CONT 1-hour post-feeding (P<.05), where it had significantly lower levels compared to CORN (figure 12). A time effect was found for eca-mir 4865p for day 28 one after feeding (P<.05), both groups had a down-regulation of this miRNA at this time point compared to day 0. Although not a significant difference five of the 12 miRNAs that had a treatment effect (eca-mir 4865p, eca-mir 598, eca-mir 195, eca-mir 192, eca-mir 129a5p) had higher levels in CORN compared CONT on Day 28 at one-hour post-feeding.

Analysis of equine skeletal muscle for 16 miRNAs found in all horses at every time point found three miRNAs (eca-mir 1515p, eca-mir 106b, and eca-mir 133a) to have a treatment effect post corn supplementation (P<.05) and 3 miRNAs (eca-mir 129a5p, eca-mir 191, eca-mir 10b) to have a trend towards a significant treatment effect (.1>P>.05) (Figure 13). No time or time by treatment effect was found.

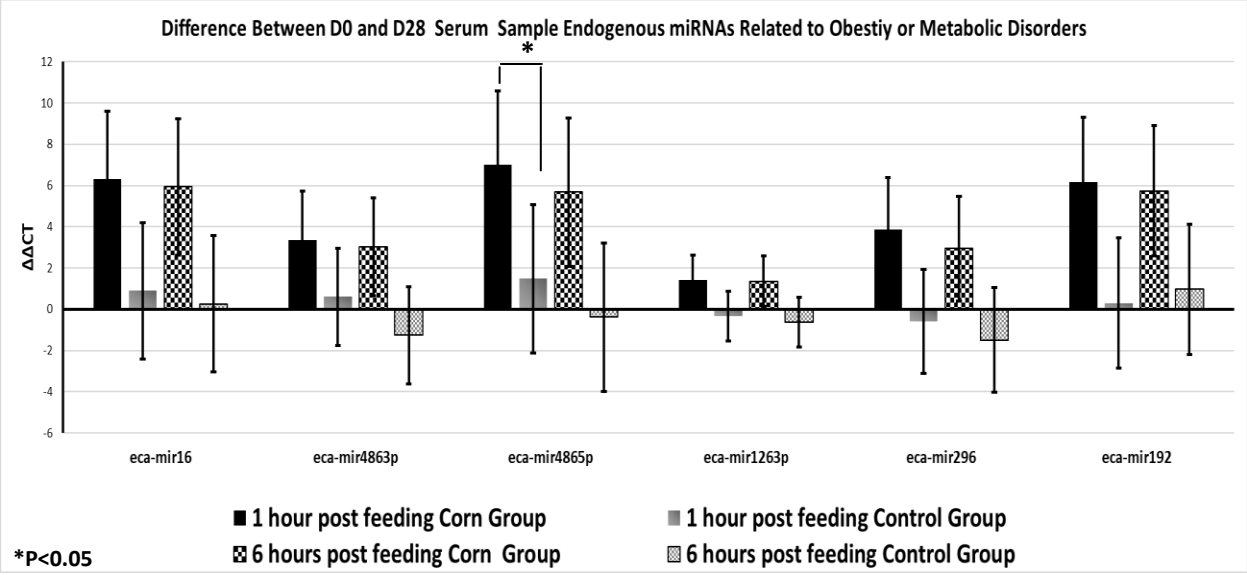

Figure 11: Difference between day 0 and day 28 Serum Sample Endogenous miRNAs Related to Obesity or Metabolic Disorders. qRT-PCR was performed to determine levels of endogenous miRNAs in serum across both diets at day 0 and day 28 1 hour and 6 hours post-feeding. Endogenous miRNAs associated with Obesity or Metabolic disorders with a significant treatment effect (P<.05) in serum represented as the mean difference between

baseline and Day 28 1-hour post-feeding and 6 hours post-feeding with 95% CI. *Eca-mir486-5p also had a time effect 1-hour post-feeding corn (P<.05). No time by treatment effect was found for these miRNAs.

Table 3: Difference in Endogenous miRNAs within Serum from Day 0 to Day 28. qRT-PCR was performed to determine levels of endogenous miRNAs in serum across both diets at day 0 and day 28 1 hour and 6 hours post-feeding. Represented as the mean difference between baseline and Day 28 1-hour post-feeding and 6 hours post-feeding with 95% CI. No time or time by treatment effect was found.

	Day 28 1-hour post feeding		**Day 28 6-hours post feeding**	
miRNA	**Corn Group (ΔΔCt)**	**Control Group(ΔΔCt)**	**Corn Group (ΔΔCt)**	**Control Group (ΔΔCt)**
ecamir598	2.36±1.25	0.95±1.25	2.86±1.25	0.64±1.25
ecamir326	5.39±3.40	0.04±3.40	4.78±3.40	2.67±3.40
ecamir3715p	2.89±3.02	-1.42±3.02	2.21±3.02	0.64±3.02
ecamir1271	2.74±2.32	-1.62±2.32	2.66±2.32	-2.51±2.32
ecamir195	7.99±4.28	0.63±4.28	7.48±4.28	-0.98±4.28
ecamir4903p	-1.17±3.11	-5.26±3.11	-1.11±3.11	-4.69±3.11

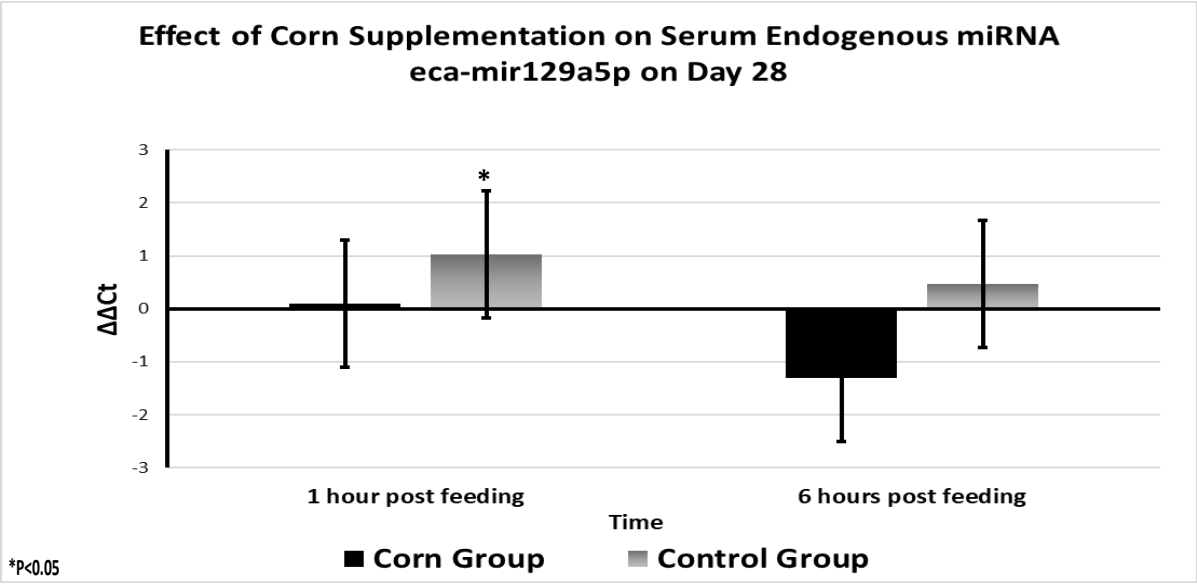

Figure 12:Effect of Corn Supplementation on Serum Endogenous miRNA eca-mir129a5p. qRT-PCR was performed to determine levels of endogenous miRNAs in serum across both diets at day 0 and day 28 1 hour and 6 hours post-feeding. Eca-mir129a5p had a significant time by treatment effect for the control at 1 hour (P<.05).

Represented as the mean difference between baseline and Day 28 1-hour post-feeding and 6 hours post-feeding with 95% CI. No treatment or time effect was found.

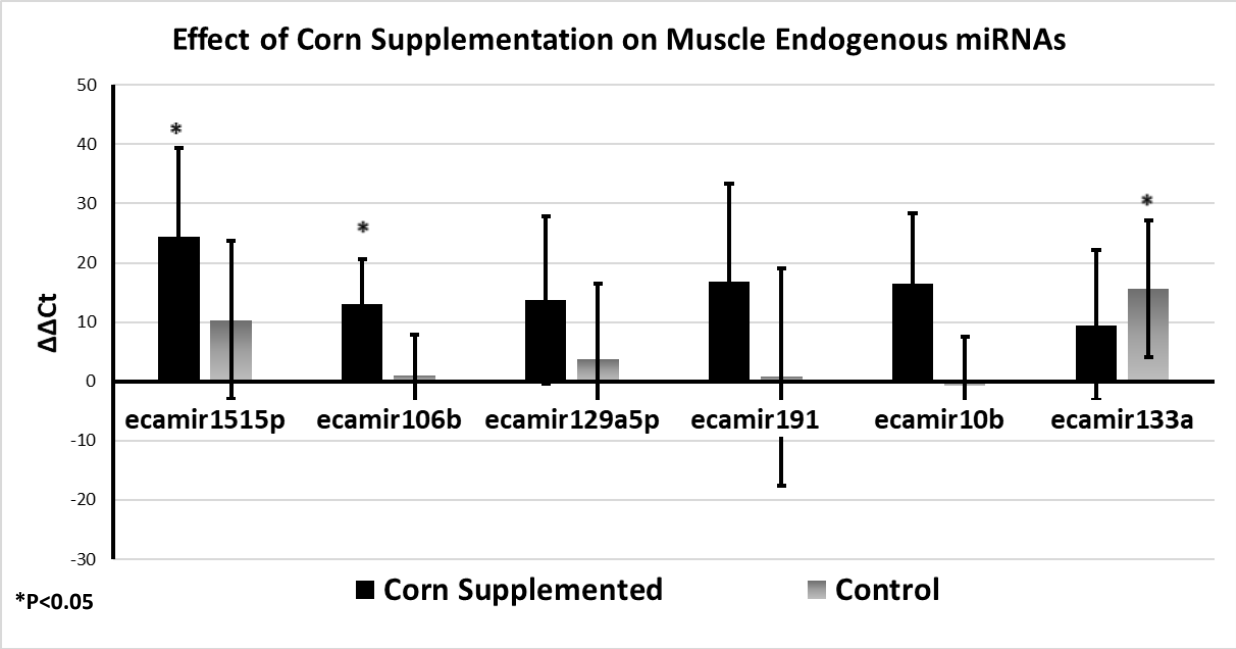

Figure 13:Effect of Corn Supplementation on Muscle Endogenous miRNAs. qRT-PCR was performed to determine levels of endogenous miRNAs in muscle across both diets at day 0 and day 28. Eca-mir1515p, -106b, and -133a had a significant treatment effect (P<.05). Represented as the mean difference between baseline and Day 28 with 95% CI. No time effect or time by treatment effect was found.

DISCUSSION

To determine how diet impacts endogenous miRNAs half of the horses (n=6) in our study were supplemented with corn for 28 days with serum samples being taken pre and post supplementation for comparison. Of the 277 miRNAs analyzed by qRT-PCR, 41 were found to appear in every horse at every time point and used for statistical analysis. Twelve miRNAs were found to have a treatment effect of which six, eca-mir16, -4863p, -4865p, -126-3p, -296, and -192, have been linked to obesity or metabolic disease (Figure 11). Meerson et al. found mir16-2-3p and mir126-5p could be used distinguish early from complicated T2DM in individuals with 77% accuracy, these two miRNAs are in the same family as mir16 and mir126-3p respectively suggesting that these families could serve as biomarkers for metabolic diseases involving similar

components as T2DM (Meerson et al., 2019). Mir 126-3p has also been found to be upregulated in patients with diabetic kidney disease (Assmann et al., 2018), have decreased levels in plasma and circulating angiogenic cells of patients with T2DM compared to healthy individuals, however amongst human subjects with T2DM those with a history of a major cardiac event had lower levels of mir126-3p, suggests it can serve as a biomarker for systemic inflammation and angiogenic status (Olivieri et al., 2015). Within our study we found the equine variant of mir126-3p to be downregulated and have lower levels on day 28 in CORN compared to CONT which could suggest based on Olivieri et al.'s study that a diet supplemented with corn increases the chances of horses developing metabolic disease. We found eca-mir4863p to be downregulated form D0 and at similar or lower levels in CORN compared to CONT, this is in contrast to Nulton's study which found this miRNA to be increased in horses fed corn (Nulton 2014), in addition to previous studies the have found circulating mir4863p in humans to be increased in T2DM patients and obese children (Meerson et al., 2019, Marzano et al., 2018, Prats-Puig et al., 2013). Within our study we observed higher circulating levels of eca-mir4865p in CORN compared to CONT on day 28 1 hour post-feeding, there was not a significant difference in the level within circulation between the groups but this observation is notable as mir4865p has previously been found to be present in higher levels in circulation within humans studies looking at pre-pubescent obesity and males with metabolic syndrome (Prats-Puig et al., 2013, Zaki et al., 2019). Compared to D0 both groups had downregulation of mir4865p at 1-hour post-feeding on day 28 explaining the time effect that was found and the treatment effect is illustrated by CORN having more downregulation of this miRNA than CONT(figure 11). The next miRNA in serum that was found to be impacted by corn supplementation was eca-mir296, studies conducted in humans have illustrated an association between mir296 and regulation of adipose tissue, with

higher concentrations being found in the visceral adipose tissue of obese individuals and amongst obese individuals, those with T2DM had lower expression (Gentile et al., 2018). Although the Gentile et al. study linking mir296 to the regulation of adipose tissue was looking at miRNA levels within tissue and did not investigate circulating miRNAs, it investigated both visceral and subcutaneous adipose tissue which would require some form of non-paracellular communication as these two tissues are found in different areas of the body, the circulatory system is often utilized in this process, suggesting the possibility that circulating levels of mir296 could also differ, especially during adipogenesis. Another miRNA found within our study linked to obesity and related disorders was mir192, within our study we found the miRNA to be downregulated in CORN compared to Day 0; however, circulating levels of this miRNA in CORN on day 28 at 1 hour was higher than CONT a notable observation but a significant difference did not exist. Previous studies have illustrated the possibility of mir192 as a conserved biomarker for obesity and metabolic states as it was found to have increased levels in the circulation in both mice and humans suffering from obesity and metabolic disorders (Jones et al., 2017. Ortega et al., 2013). Multiple studies have also found increased levels of mir-192 in exosomes of individuals with insulin resistance, suggesting it plays a role in the problem(Castano et al., 2018, Parrizas et al., 2015, Shah et al., 2017, Jones et al., 2017). Mir192-5p which is in the same family as mir192 has been found to regulate lipid synthesis in non-alcoholic fatty liver disease and to be downregulated in patients with diabetic-related kidney disease(Liu, X-L et al., 2017, Assmann et al., 2018). The studies associating mir192 with obesity, metabolic disorders, insulin resistance, and a possible role in lipid synthesis suggest that mir192 should be included in future studies looking at these disorders across species.

When searching through previous studies to determine if miRNAs found to have a treatment effect related to obesity and related metabolic diseases which in horses have been associated with diets high in NSC, eca-mir326 was not found to have an impact directly on these conditions and so was not included in figure 11; However, mir326 has been linked to reduced inflammation in pulmonary fibrosis when upregulation occurs (Xu et al., 2019). The association between reduction of inflammation suggests mir326 plays a role in regulating at least one pathway of inflammation and could impact inflammation due to other causes throughout the body. Within our study, mir326 was downregulated significantly in CORN horses on day 28 and had a larger downregulation than CONT (table 3), also lower circulating levels were observed in CORN compared to CONT suggesting that CORN may have had the potential for higher levels of inflammation. As obesity is considered to be a chronic state of inflammation, future studies looking at levels of mir326 in obese individuals could identify an association between levels of mir326 and obesity.

Obesity is not usually associated with Type 1 Diabetes mellitus (T1DM), which like T2DM causes high glucose concentrations in blood, but unlike T2DM where the high glucose is a result of insulin resistance in tissues preventing insulin-dependent glucose uptake form occurring T1DM is associated with a lack of insulin being produced by the beta-cells within the pancreas. A potential miRNA biomarker for T1DM has been identified in mir326, as it is downregulated in individuals with T1DM (Azhir et al., 2019, Garcia-Diaz et al., 2018). Type 1 diabetes has been linked to an increased risk of heart disease usually characterized by cardiac hypertrophy, silencing mir-195, which was found in our study to be downregulated in CORN horses after supplementation (table 3), has been shown to reduce myocardial hypertrophy,

improve coronary blood flow, and myocardial function in mice with diabetes, suggesting mir-195 could serve as a novel therapy for diabetic heart diseases (Zheng et al., 2015).

Eca-mir129a5p was observed to have higher levels on day 28 in CORN compared to the CONT horses at 1-hour post-feeding, not a significant difference, and was upregulated compared to day 0 in CORN. It has been proposed that mir129a3p, which is in the same family as mir129a5p, plays a role in glucose and lipid metabolism (Pescador et al.,2013), and mir129a5p specifically been shown to regulate glycolysis and cell proliferation by targeting glucose transporters on gastric cancer cells causing reduced glucose uptake and consumption (Chen et al., 2018) demonstrating it can affect glucose utilization and could potentially also impact normal gastric cells. Eca-mir129a5p was found to have higher levels in circulation within insulin resistant horses compared to insulin-sensitive horses (Santos et al., 2018), we observed higher levels in CORN suggesting they may have been at a higher risk of developing insulin resistance as a result of corn supplementation, but as previously stated there was not a significant difference in levels between the groups (Santos et al., 2018). Further studies looking at if mir129a5p affects glucose utilization of normal cells and how it is impacted by a meal could provide more information on how diet and genes interact within the body. Within horses specifically more studies should be conducted within normal and insulin-resistant horses, potentially on a variety of diets, to see how this particular miRNA is impacted by diet.

The impact of diet on overall health has been investigated in horses for many years, but only one other study has looked at how diet impacts equine endogenous miRNAs specifically. In Nulton's study, three different diets were given: alfalfa diet, alfalfa and corn diet, and alfalfa and rice diet to investigate how endogenous miRNA profiles in serum would change within horses given the different diets (Nulton 2014). In our study, we wanted to further investigate how a diet

supplemented with corn impacts endogenous miRNAs in equine serum and muscle. Unlike Nulton's study which took serum samples six hours post-feeding, we decided to take serum samples every fifteen minutes up to two hours post-feeding, we only analyzed D0 and D28 1 hour and 6 hours post-feeding samples due to time and material constraints, but future studies could test the stored serum samples to create a better picture of how endogenous miRNAs within serum vary post-meal. Our study only looked at circulating levels of miRNAs in total serum and did not look at the profiles of circulating miRNAs in exosomes, the possibility exists that the profiles of miRNAs within exosomes differed from that of total serum. We did not find any members of the *Let-7* miRNA family to be significantly impacted by corn supplementation even though previous studies have linked them to obesity and T2DM in humans (Gentile et al., 2018) and found lower levels in serum exosomes after horses were fed corn (Nulton, 2014). The *Let*-7 family has been shown to have a strong association with glucose tolerance and insulin sensitivity (Rottiers and Näär, 2012)., the absence of any significant changes in these miRNAs could be attributed to the level of corn being fed to the horses. Within this study, horses were fed 1 lb. of steam flaked corn a day, whereas in Nulton's study they received 2 lbs. of kibbled corn feed, composed of 80% corn and 20% wheat resulting in roughly 1.6 pounds of corn a day (Nulton, 2014). The difference in feed processing and composition between our study and Nulton's could have impacted the digestibility of the corn and how the nutrients were metabolized. Analysis of the steam flaked corn fed in our study found it contained 78.6 % starch and 1.5% simple sugars (Appendix Table III-A), the two main groups within non-structural carbohydrates, Nulton's study does not appear to have analyzed the feed for these specific components making it impossible to compare these particular feed components between the studies to determine if the difference in serum results could be attributed to nutritional makeup of feeds. More studies

looking at the impact of diet on circulating miRNA profiles in horses should be conducted to create a better picture of the interaction between diet and the overall health of horses.

After completion of evaluating skeletal muscle for 277 equine miRNAs was completed, 16 miRNAs were found to consistently appear within the equine muscle, with some miRNAs usually expressed in muscle not appearing within our study. Due to the low number of miRNAs appearing in every horse at every time point some miRNAs that appeared in the majority of horses at all time points were included to reach the 16 total miRNAs that were used for statistical analysis. Muscle samples were the first to be evaluated with qRT-PCR and after all 24 samples (12 from D0 and 12 from D28) had been analyzed in duplicate for all 277 equine miRNAs and 1 housekeeping gene it was discovered that the endogenous primers had only been diluted to 100µM instead of the intended 10µM, this is extremely likely to have resulted in the small number of miRNAs meeting analysis criteria. The primers were properly diluted for all of the serum evaluations. We had intended to reanalyze the muscle samples after all serum evaluations were completed but a lack of some reagents, time, and funds prevented analysis to be reconducted on the muscle samples.

Statistical analysis of the 16 miRNAs selected for appearing in most samples found eca-mir1515p, eca-mir106b, and eca-mir133a to have a significant treatment effect ($P<.05$), all of which have been linked to obesity or muscle insulin response. Within our study eca-mir1515p was found to be significantly Downregulated in CORN compared to D0 and CONT, mir1515p is differently expressed in humans considered obese compared to normal individuals, suggesting expression of this miRNA either plays a role in the regulation of obesity or is regulated itself by the process involved in obesity (Arner et al., 2012). Skeletal muscle is the main tissue responsible for post-prandial glucose uptake and over 70% of insulin-dependent glucose uptake

from the blood. Skeletal muscle insulin resistance has been demonstrated in individuals with T2DM and plays a role in the development of cardiovascular diseases (Ferland-McCollough et al., 2010, Gallagher et al., 2010). Within insulin-resistant skeletal muscle, mir106b was found to be upregulated and contribute to the regulation of skeletal muscle glucose uptake (Zheng, L-F. et al., 2019). Regulation of glucose uptake by this miRNA is due to targeting genes coding for mitofusin-2 (Mfn2) and glucose transporter (GLUT)-4 resulting in reduced levels of these proteins, reduced insulin sensitivity, and increased blood glucose concentrations. (Zhang Y et al., 2013, Zhang Y et al., 2017). The findings of our study that eca-mir106b was downregulated in CORN after supplementation and compared to CONT, suggests that the amount of corn supplemented did not cause the muscle group samples to begin to develop or develop insulin resistance.

Previous studies of muscle across species show higher levels of mir133a in muscle tissue compared to other tissues and have shown it plays a role in myogenesis and regeneration (Rao et al., 2006). Within skeletal muscle, mir133a plays an important role in mitochondrial biogenesis and was found to be downregulated in skeletal muscle with insulin resistance and samples from T2DM patients (Nie et al. 2016, Zheng, L-F. et al., 2019, Gallagher et al., 2010). Within our study we did find mir133a to be downregulated in skeletal muscle on D28 compared to D0 in CORN, however, this miRNA had a more significant downregulation in CONT which is in contrast to the previously mentioned studies examining this miRNA (Figure 13). The error that occurred with the concentration of primer very likely resulted in fewer miRNAs being identified as having significant changes as too much primer can prevent detection of SYBER green, as a result, we decided to also look at miRNAs that had a trend for significance to determine if any had associations with obesity, insulin resistance, or associated diseases.

A trend toward a significant treatment effect was found for eca-mi10b, eca-mir129a5-, and eca-mir191 (.05<P<.1) within skeletal muscle. Mir10b was found to be downregulated in CORN compared to D0 while remaining close to the same in CONT, this miRNA has been linked to T2DM, with downregulation noted in muscle tissue of hyperglycemic mice (Herrera et al., 2010). As previously mentioned mir129a5p has been shown to regulate glycolysis within gastric cancer cells causing reduced glucose uptake and consumption (Chen et al., 2018), within our study we found this miRNA to be downregulated within CORN after supplementation. Considering the other miRNAs examined like mir106b which suggests the muscle tissue sampled was not insulin resistant, it would make sense that a miRNA responsible for reduced glucose intake would be downregulated when horses are feeding a diet higher in readily available glucose in the form of NSCs. A review of previous studies did not show any association between mir191 and obesity or related diseases.

As previously mentioned the *Let-7* miRNA family has been strongly associated with diabetes when found in circulation and adipose tissue. This family of miRNA is also well known to act as a regulator of glucose within skeletal muscle with glucose intolerance and increased fat resulting from widespread overexpression of let-7 (Frost and Olson, 2011). No changes were identified within our study for let-7 within skeletal muscle samples, but future studies looking at how this family is specifically impacted by diet would be beneficial in aiding to evaluate them as potential biomarkers for early detection of glucose intolerance in horses and aid in early diet interventions. Our study is the first equine study to look at the impact of diet on skeletal muscle miRNA profiles, as Nulton did not analyze tissue samples for endogenous miRNAs.

Our study illustrates that diet does have a role in regulating levels of endogenous miRNAs in circulation and skeletal muscle of horses. The differing results we found for miRNAs

related to obesity and insulin resistance within our study compared to other studies could be due to all horses used within the study having no known history of insulin resistance, obesity, or EMS, whereas many of the studies linking miRNAs to conditions like obesity, insulin resistance, and diabetes utilized individuals already diagnosed with these conditions. The differences in upregulation or downregulation between our study and others should not be dismissed, but the ability of the corn supplemented diet to cause changes in expression levels of these miRNAs should also be recognized as it suggests the possibility that long term consumption of this diet could lead to more dramatic changes in miRNA expression more closely resembling the other studies. It should also be noted that making comparisons between studies can be difficult as the physiology of horses differs from humans and mice, this could also contribute to differences in findings. Despite multiple studies evaluating miRNA expression in serum and skeletal muscle of diabetic and insulin-resistant individuals, there are not many studies indicating exactly how these miRNAs are regulated and expression of miRNAs within serum and muscle likely involves complex crossovers of various metabolic pathways. Additional studies need to be conducted to better determine the physiologic role of skeletal muscle and serum miRNA expression in insulin resistance and metabolic disorders like EMS in horses. Future studies utilizing various diets should also be conducted and could serve to better illustrate and make associations between diet and changes in miRNAs related to obesity and insulin resistance within horses.

CHAPTER IV: DISCUSSION AND CONCLUSIONS

Based upon the results from the first objective of this study we can conclude that plant miRNAs can be absorbed, released into circulation, and taken up by muscle tissue in horses. These findings suggest the possibility of interspecies gene regulation. The ability of diet-derived miRNAs to be absorbed and identified within total serum and muscle tissue is a major step in the process of gaining a better understanding of the mechanisms involved in the uptake of diet-derived miRNAs and the roles they may play in gene regulation within animals. Additionally, this study is one of the first to demonstrate the ability of diet to impact endogenous miRNAs in serum and the first to demonstrate the impact on endogenous miRNAs in muscle. The potential for miRNAs within serum and muscle to be used as biomarkers for the detection of disease and nutritional status of horses could allow for earlier and more cost-effective detection of disease. This study aimed to identify the presence of diet-derived plant miRNAs within equine serum and muscle and to examine the effect of a corn supplemented diet on profiles of endogenous miRNAs in circulation and muscle.

These findings add support to the hypothesis that diet-derived plant miRNAs, including corn-based miRNAs, are absorbed from the digestive tract and hold the potential via translational repression of endogenous mRNAs to regulate endogenous gene expression. Based on this study there is not enough evidence to suggest that the presence of the corn miRNA (zma-miR827-5p) would cause changes in gene expression as zma-miR827-5p does not currently have any mammalian gene targets identified. As mammalian gene targets are identified for more plant miRNAs they will share targets with endogenous miRNAs and could emerge as competitors for binding to the targets changing overall gene expression. Our study was able to illustrate how

circulating levels of diet-derived miRNAs varied after ingestion of food over two hours and 6 hours after ingesting corn. The results showed levels of diet-derived miRNAs in the corn feed (zma-miR827-5p) spiked 15 minutes after feeding corn to horses then began to vary in their appearance, the levels of ath-156a and osa-mir1866-3p also varied thought out the sampling window but to a lesser degree. The variation in the detection of these miRNAs could be a result of uptake into tissue, dilution within circulation, or incorporation into exosomes. Our study was not able to illustrate any changes in levels of plant miRNAs within tissue of horses, this could be due to multiple factors including insufficient levels of corn supplemented, changes occurring in other tissues, and it is unknown how long plant miRNAs remain detectable in skeletal muscle. Additionally, differences in digestion/metabolism of animal models need to be investigated and better normalizers developed or identified to be used within horses.

Examination of serum and muscle samples at day 28 revealed several miRNAs that were differentially expressed from day 0 after corn supplementation. 11 miRNAs were differentially expressed in the serum of the horses fed a diet supplemented with corn on day 28 compared to day 0. The majority of these miRNAs have previously been reported to be linked with obesity, diabetes, and insulin resistance. 1 miRNA was found to be upregulated significantly in the corn group compared to the control group 1-hour post-feeding and previously was reported to be higher in insulin-resistant horses in addition to playing a role in glucose metabolism. Three miRNAs were differentially expressed in muscle tissue of horses fed corn, all of which have previous reports of association with obesity, insulin resistance, or diabetes. The dysregulation of these miRNAs within serum and muscle suggests that diets high in sugar and starch, like corn, initiate changes in miRNA expression that could lead to insulin resistance and obesity, two major components of Equine Metabolic Syndrome. The findings of this study contribute to developing

a better understanding of the role diet has to play in the management of equine metabolic diseases.

Finally, this data supports the belief that diet plays a major role in the expression of endogenous miRNAs and the potential for diet-derived miRNAs to regulate mRNA expression within mammals. Further research should be conducted to determine mechanisms by which diet-derived miRNAs are taken up into circulation in tissues and if they have a functional impact on endogenous gene expression. More studies should also be conducted within horses to examine the interaction between diet and genes linked to metabolic diseases, as the frequency of these diseases is increasing in the horse population.

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APPENDICES

Appendix Table I: 277 Equine miRNA primer sequences

Mature miRNA ID	Target miRNA Mature Sequence
eca-let-7a	ugagguaguagguuguauaguu
eca-let-7c	ugagguaguagguuguaugguu
eca-let-7d	agagguaguagguugcauaguu
eca-let-7e	ugagguaggagguuguauaguu
eca-let-7f	ugagguaguagauuguauaguu
eca-let-7g	ugagguaguaguuuguacaguu
eca-mir-7	uggaagacuagugauuuuguugu
eca-mir-9a	ucuuugguuaucuagcuguauga
eca-mir-10a	uacccuguagauccgaauuugug
eca-mir-10b	uacccuguagaaccgaauuugug
eca-mir-15a	uagcagcacauaaugguuugug
eca-mir-15b	uagcagcacaucaugguuuaca
eca-mir-16	uagcagcacguaaauauuggcg
eca-mir-17	caaagugcuuacagugcagguag
eca-mir-18b	uaaggugcaucuagugcaguuag
eca-mir-19a	ugugcaaaucuaugcaaaacuga
eca-mir-19b	ugugcaaaucuaugcaaaacuga
eca-mir-20a	uaaagugcuuauagugcagguag
eca-mir-20b	caaagugcucauagugcagguag
eca-mir-21	uagcuuaucagacugauguuga
eca-mir-22	aagcugccaguugaagaacugu
eca-mir-23a	aucacauugccagggauuucc
eca-mir-23b	aucacauugccagggauuacc
eca-mir-24	uggcucaguucagcaggaacag
eca-mir-25	cauugcacuugucucggucuga
eca-mir-26a	uucaaguaauccaggauaggcu
eca-mir-27a	uucacaguggcuaaguuccgc
eca-mir-27b	uucacaguggcuaaguucugc
eca-mir-283p	cacuagauugugagcuccugga
eca-mir-285p	aaggagcucacagucuauugag
eca-mir-29a	uagcaccaucugaaaucgguua
eca-mir-29c	uagcaccauuugaaaucgguua
eca-mir-30b	uguaaacauccuacacucagcu
eca-mir-30c	uguaaacauccuacacucucagc
eca-mir-30e	uguaaacauccuugacuggaag
eca-mir-31	aggcaagaugcuggcauagcu
eca-mir-32	uauugcacauuacuaaguugca

eca-mir-34	uggcagugucuuagcugguugu
eca-mir-92a	uauugcacuugucccggccugu
eca-mir-92b	uauugcacucgucccggccucc
eca-mir-93	caaagugcuguucgugcagguag
eca-mir-96	uuuggcacuagcacauuuuugcu
eca-mir-98	ugagguaguaaguuguauuguu
eca-mir-99a	aacccguagauccgaucuugug
eca-mir-99b	cacccguagaaccgaccuugcg
eca-mir-100	aacccguagauccgaacuugug
eca-mir-101	uacaguacugugauaacugaa
eca-mir-103	agcagcauuguacagggcuauga
eca-mir-105	ucaaaugcucagacuccuguggu
eca-mir-106a	caaagugcuuacagugcagguag
eca-mir-106b	uaaagugcugacagugcagau
eca-mir-107b	agcagcauuguacagggcuauca
eca-mir-122	uggagugugacaaugguguuug
eca-mir-125a5p	ucccugagacccuuuaaccuguga
eca-mir-125b	ucccugagacccuaacuuguga
eca-mir-1263p	ucguaccgugaguaauaaugcg
eca-mir-127	ucggauccgucugagcuuggcu
eca-mir-128	ucacagugaaccggucucuuu
eca-mir-129a3p	aagcccuuaccccaaaaaguau
eca-mir-129a5p	cuuuuugcggucugggcuugc
eca-mir-132	uaacagucuacagccauggucg
eca-mir-133a	uuuggucccccuucaaccagcug
eca-mir-133b	uuuggucccccuucaaccagcua
eca-mir-134	ugugacugguugaccagagggg
eca-mir-135a	uauggcuuuuuauuccuauguga
eca-mir-135b	uauggcuuuucauuccuauguga
eca-mir-136	acuccauuuguuuugaugaugg
eca-mir-137	uuauugcuuaagaauacgcguag
eca-mir-138	agcugguguugugaaucaggccg
eca-mir-1393p	ggagacgcggcccuguuggagu
eca-mir-1395p	ucuacagugcacgugucuccag
eca-mir-1403p	uaccacaggguagaaccacgg
eca-mir-1405p	cagugguuuuacccuaugguag
eca-mir-141	uaacacugucugguaaagaugg
eca-mir-1423p	uguaguguuuccuacuuuaugga
eca-mir-143	ugagaugaagcacuguagcuc
eca-mir-144	uacaguauagaugauguacu
eca-mir-145	guccaguuuucccaggaaucccu
eca-mir-146a	ugagaacugaauuccauggguu
eca-mir-146b3p	ugcccuagggacucaguucugg
eca-mir-146b5p	ugagaacugaauuccauaggcu
eca-mir-147b	gugugccgaaaugcuucugcua

eca-mir-148a	ucagugcacuacagaacuuugu
eca-mir-148b3p	ucagugcaucacagaacuuugu
eca-mir-149	ucuggcuccgugucuucacuccc
eca-mir-150	ucucccaacccuuguaccagug
eca-mir-1515p	ucgaggagcucacagucuagu
eca-mir-153	uugcauagucacaaaagugauc
eca-mir-154	uagguuauccguguugccuucg
eca-mir-155	uuaaugcuaaucgugauaggggu
eca-mir-181a	aacauucaacgcugucggugagu
eca-mir-181b	aacauucauugcugucggugggu
eca-mir-182	uuuggcaaugguagaacucacacug
eca-mir-184	uggacggagaacugauaagggu
eca-mir-186	caaagaauucuccuuuugggcu
eca-mir-187	ucgugucuuguguugcagccgg
eca-mir-1883p	cucccacaugcaggguuugca
eca-mir-1885p	caucccuugcaugguggaggg
eca-mir-190b	ugauauguuugauauuggguu
eca-mir-191	caacggaaucccaaaagcagcug
eca-mir-192	cugaccuaugaauugacagcc
eca-mir-193a5p	uggguuuugcgggcgagauga
eca-mir-193b	aacuggcccacaaagucccgcu
eca-mir-194	uguaacagcaacuccaugugga
eca-mir-195	uagcagcacagaaauauuggc
eca-mir-196a	uagguaguuucauguuguuggg
eca-mir-196b	uagguaguuuccuguuguuggg
eca-mir-197	uucaccaccuucuccacccagc
eca-mir-199b3p	acaguagucugcacauugguua
eca-mir-199b5p	cccaguguuuagacuaucuguuc
eca-mir-200b	uaauacugccugguaaugauga
eca-mir-200c	uaauacugccggguaaugaugga
eca-mir-204b	uucccuuugucauccuaugccu
eca-mir-205	uccuucauuccaccggagucug
eca-mir-206	uggaauguaaggaagugugugg
eca-mir-211	uucccuuugucauccuuugccu
eca-mir-215	augaccuaugaauugacagac
eca-mir-216a	uaaucucagcuggcaacuguga
eca-mir-216b	aaaucucugcaggcaaauguga
eca-mir-217	uacugcaucaggaacugauugga
eca-mir-218	uugugcuugaucuaaccaugu
eca-mir-221	agcuacauugucugcuggguuuc
eca-mir-222	agcuacaucuggcuacugggu
eca-mir-223	ugucaguuugucaaauacccca
eca-mir-224	caagucacuagugguuccguu
eca-mir-296	gaggguuggguggaggcuuucc
eca-mir-301b3p	cagugcaaugauauugucaaagc

eca-mir-302a	uaagugcuuccauguuuuaguga
eca-mir-302b	uaagugcuuccauguuuuaguag
eca-mir-302c	uaagugcuuccauguuucagugg
eca-mir-302d	uaagugcuuccauguuuuagugu
eca-mir-3233p	cacauuacacggucgaccucu
eca-mir-3235p	aggugguccguggcgcguucgc
eca-mir-3245p	cgcaucccccuagggcauuggugu
eca-mir-326	ccucugggcccuuccuccagc
eca-mir-330	ucucugggccuguguucuuaggc
eca-mir-331	gccccugggccuauccuagaa
eca-mir-335	ucaagagcaauaacgaaaaaugu
eca-mir-3373p	cuccuaugagaugccuuuccuc
eca-mir-3375p	gaacggcuucauacaggagcu
eca-mir-3383p	uccagcaucagugauuuuguug
eca-mir-3385p	aacaauauccuggugcugagug
eca-mir-3405p	uuauaaagcaaugagacugauu
eca-mir-3423p	ucucacacagaaaucgcacccgu
eca-mir-3455p	gcugacuccuaguccagugcuc
eca-mir-346	ugucugcccgcaugccugccucu
eca-mir-3613p	ucccccaggcgugauucugauuu
eca-mir-3615p	uuaucagaaucuccagggguac
eca-mir-3623p	aacacaccuauucaaggauuca
eca-mir-3625p	aauccuuggaaccuaggugugagu
eca-mir-3693p	aauaauacaugguugaucuuu
eca-mir-3695p	agaucgaccgugucauauucgc
eca-mir-370	gccugcugggguggaaccuggu
eca-mir-3715p	acucaaacuguggggcacu
eca-mir-374b	auauaauacaaccugcuaagug
eca-mir-376c	aacauagaggaaauuccacgu
eca-mir-378	acuggacuuggagucagaagg
eca-mir-379	ugguagacuauggaacguagg
eca-mir-380	uauguaauaugguccacgucuu
eca-mir-381	uauacaagggcaagcucucugu
eca-mir-382	gaaguuguucgugguggauucg
eca-mir-383	agaucagaaggugauuguggcu
eca-mir-4093p	gaauguugcucggugaaccccu
eca-mir-4095p	agguuacccgagcaacuuugcau
eca-mir-411	uaguagaccguauagcguacg
eca-mir-412	uucaccugguccacuagccg
eca-mir-421	ggccucauuaaauguuuguug
eca-mir-4235p	ugaggggcagagagcgagacuuu
eca-mir-424	cagcagcaauucauguuuugaa
eca-mir-429	uaauacugucugguaaugccg
eca-mir-431	ugucuugcaggccgucaugcagg
eca-mir-432	ucuuggaguaggucauugggugg

eca-mir-433	aucaugaugggcuccucggugu
eca-mir-448	uugcauauguaggaugucccau
eca-mir-449a	uggcaguguauuguuagcuggu
eca-mir-450b5p	uuuugcaauauguuccugaaua
eca-mir-451	aaaccguuaccauuacuguguu
eca-mir-454	uagugcaauauugcuuauagggu
eca-mir-4853p	gucauacacggcucuccucucu
eca-mir-4855p	agaggcuggccgugaugaauuc
eca-mir-4863p	cggggcagcucaguacaggau
eca-mir-4865p	uccuguacugagcugccccgag
eca-mir-487b	aaucguacagggucauccacuu
eca-mir-488	uugaaaggcuauuucuugguc
eca-mir-489	gugacaucacauauacggcggc
eca-mir-4903p	caaccuggaggacuccaugcug
eca-mir-4905p	ccauggaucuccaggugggu
eca-mir-4913p	cuuaugcaagauucccuucuac
eca-mir-4915p	agugggaacccuuccaugagg
eca-mir-493b	ugaaggucuuccgugugccagg
eca-mir-494	ugaaacauacacgggaaaccuc
eca-mir-495	aaacaaacauggugcacuucuu
eca-mir-497	cagcagcacacugugguuugu
eca-mir-4993p	aacaucacagcaagucugugcu
eca-mir-500	uaauccuugcuaccugggugaga
eca-mir-501	auccuucgucccugggugaga
eca-mir-5023p	aaugcaccugggcaaggauuca
eca-mir-5025p	auccuugcuaucugggugcua
eca-mir-503	uagcagcgggaacaguacugcag
eca-mir-504	agacccuggucugcacucuauc
eca-mir-505	cgucaacacuugcugguuuccu
eca-mir-507	auuggcaccucuuagagugaa
eca-mir-5083p	ugauugucaccuuuuggaguaga
eca-mir-5085p	uacuccagagggugucauucaca
eca-mir-5095p	uacugcagacaguggcaauca
eca-mir-514	auugacaccucugugagugga
eca-mir-5325p	caugccuugaguguaggaccgu
eca-mir-541	ugggugggcacagaauccagucu
eca-mir-5423p	ugugacagauugauaacugaaa
eca-mir-5425p	cucggggaucaucaugucacga
eca-mir-544b	auucugcauuuuuaacaaguuc
eca-mir-545	ucaacaaacauuuauugugugc
eca-mir-551a	gcgacccacucuugguuucca
eca-mir-551b	gcgacccauacuugguuucag
eca-mir-5823p	uaaccgguugaacaacugaacc
eca-mir-5905p	gagcuuauucauaaaaguacag
eca-mir-592	uugugucaauaugcgaugaugu

eca-mir-598	uacgucaucguugucaucguca
eca-mir-6155p	gggggucccggugcucggauc
eca-mir-6153p	uccgagccugggucucccucuc
eca-mir-628a	augcugacauauuuacuagagg
eca-mir-632	gugccuguuuccuguggga
eca-mir-652	aauggcgccacuaggguugug
eca-mir-656	aauauuauacagucaaccucu
eca-mir-660	uacccauugcauaucggaguug
eca-mir-664	uauucauuuaucuccuagccuaca
eca-mir-670	gucccugaguguauguggugaa
eca-mir-6715p	aggaagcccuggaggggcuggag
eca-mir-6713p	uccgguucucagggcuccacc
eca-mir-672	ugagguugguguacuguguguga
eca-mir-6745p	ggugcucacuuguccuccu
eca-mir-684	aguuuucccuucaauucag
eca-mir-703	aaaaccuucagaaggaaagga
eca-mir-761	gcagcagggugaaacugacaca
eca-mir-763	ccagcugggaggaaccaguggc
eca-mir-7673p	ucugcucauacuccaugguuccu
eca-mir-7695p	ggagaccucuggguucugagcu
eca-mir-7693p	cugggaucucgggggucuugguu
eca-mir-769b	ggaaaccucuggguucugagcu
eca-mir-770	agcaccacgugucugggccaug
eca-mir-872	aagguuacuuguuaguucagg
eca-mir-873	gcaggaacuugugagucuccu
eca-mir-874	cugcccuggcccgagggaccga
eca-mir-8765p	uggauuucuuugugaaucacca
eca-mir-8855p	uccauuacacuacccugccucu
eca-mir-1179	aagcauucuuucauugguugg
eca-mir-1180	uuuccggcucgagugggugugu
eca-mir-1185	agaggauacccuuuguauguu
eca-mir-1193	uaggucacccguuugacuauc
eca-mir-1197	uaggacacauggucuacuucu
eca-mir-1244	gagugguugguuuguaugagaugguu
eca-mir-1248	uccuucuuguauaagcacugugcuaaa
eca-mir-1255b	cggauaagcaaagaaaugugguu
eca-mir-1261	guggauuaggcuuuggcuu
eca-mir-1271	cuuggcaccucguaagcacuca
eca-mir-1291a	uggcccugacugaagaccagcagu
eca-mir-1291b	aggcccugaaucaagaccagcagu
eca-mir-1296	uuagggcccuggcuccaucucc
eca-mir-1298	uucauucggcuguccagaugua
eca-mir-1301	uugcagcugccugggagugauuuc
eca-mir-13021	uugggacauacuuauacuaaa
eca-mir-1302b2	uugggacauacuuauacuaga

eca-mir-1302d4	uugggacauacuuaugcuaaa
eca-mir-1302e7	uugggauauacuuauacuaaa
eca-mir-1302c5	uugcgacauacuuauacuaaa
eca-mir-1461	aucucuacggguaaguguguga
eca-mir-1468	cuccguuugccuguuuugcug
eca-mir-1839	aagguagauagaacaggucuug
eca-mir-1898	aggucaagguucacaggggauc
eca-mir-1905a	caccacgagcccuaccacgcgguag
eca-mir-1905b	caccagccccacuacgcgguag
eca-mir-1905c	caccaccagccccaccacgcgguag
eca-mir-1912	uacccagagcgugcagugugaa

Appendix Table II: Non-Equine miRNA Primer Sequences. A.) Mammalian B.) Plant

A)

Mature miRNA ID	**Target Mature miRNA Sequence**
RNU 1A	CGACTGCATAATTTGTGGTAGTGG

B)

Mature miRNA ID	**Target Mature miRNA Sequence**
ath-mir-156a	UGACAGAAGAGAGUGAGCAC
zma-mir-827-5p	UUUGUUGGUGGUCAUUUAACC
osa-mir-1866-3p	UGAAAUUCCUGUAAAAUUCUUG

Appendix Table III: Feed Analyses. Reported on a dry matter basis. A.) Steam Flaked corn B.) Hays

A)

Analysis Parameters	Steam Flaked Corn
DM (%)	86.4
DE (Mcal/kg)	3.96
CP (%)	7.5
Estimated Lysine (%)	0.21
ADF (%)	2.4
NDF (%)	6.2
WSC (%)	2.1
ESC (%)	1.5
Starch (%)	78.6
NFC (%)	80.7

Dry Matter (DM), Digestible Energy (DE), Crude Protein(CP), Acid Detergent Fiber(ADF), Neutral Detergent Fiber (NDF), WSC (Water Sol. Carbs.), ESC (Simple Sugars) (%), Non- Fiber Carb. (NFC).

B)

Analysis Parameters	Round Bale	Chopped Hay mixture
DM (%)	93.4	90.6
DE (Mcal/kg)	2.21	2.18
CP (%)	7.2	17.2
Estimated Lysine (%)	0.25	0.60
Lignin (%)	3.1	5.0
ADF (%)	35.2	34.5
NDF (%)	58.3	52.5
WSC (%)	14.0	8.7
ESC (%)	5.9	7.1
Starch (%)	0.9	0.4
NFC (%)	24.5	17.7
Crude Fat (%)	2.6	3.0
Ash (%)	7.4	9.7

Dry Matter (DM), Digestible Energy (DE), Crude Protein(CP), Acid Detergent Fiber(ADF), Neutral Detergent Fiber (NDF), WSC (Water Sol. Carbs.), ESC (Simple Sugars) (%), Non- Fiber Carb. (NFC).

Appendix Table IV: Henneke Body Condition Score Chart

Body Condition Score	Description
1 Poor	Animal extremely emaciated; spinous processes, ribs, tailhead, tuber coxae, and ischia projecting prominently; the bone structure of withers, shoulders, and neck easily noticeable; no fatty tissue can be felt.
2 Very Thin	Animal emaciated; slight fat covering over base of spinous processes; transverse processes of lumbar vertebrae feel rounded; spinous process, ribs, tailhead, tuber coxae, and ischia prominent; withers, shoulders, and neck structure faintly discernible.
3 Thin	Fat buildup about halfway on spinous processes; transverse processes cannot be felt; slight fat cover over ribs; spinous processes and ribs easily discernible; tailhead prominent, but individual vertebrae cannot be identified visually; tuber coxae appear rounded but easily discernible; tuber ischii not distinguishable; withers, shoulders and neck accentuated.
4 Moderately Thin	Slight ridge along the back; faint outline of ribs discernible; tailhead prominence depends on conformation, fat can be felt around it; tuber coxae not discernible; withers, shoulder, and neck not obviously thin.
5 Moderate	Back is flat (no crease or ridge); ribs not visually distinguishable but easily felt; fat around tailhead beginning to feel spongy; withers appear rounded over spinous processes; shoulder and neck blend smoothly into body.
6 Moderately Fleshy	May have slight crease down back; fat over ribs spongy; fat around tailhead soft; fat beginning to be deposited along the side of withers, behind shoulders and along the side of neck.
7 Fleshy	May have crease down back; individual ribs can be felt, but noticeable filling

	between ribs with fat around tailhead soft; fat deposited along withers, behind shoulders, and along neck.
8 Fat	Crease down back; difficult to feel ribs; fat around tailhead very soft; area along withers filled with fat; area behind shoulder filled with fat; noticeable thickening of neck; fat deposited along inner thighs.
9 Extremely fat	Obvious crease down back; patchy fat appearing over ribs; bulging fat around tailhead, along withers, behind shoulders, and along neck; fat along inner thighs may rub together; flank filled with fat.

Original Source: Henneke et. al, 1983.